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# **D6 in cutaneous pathology**

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## ABSTRACT

Chemokines are central to the migration of leukocytes around the body, during both inflammatory and homeostatic conditions. Chemokines mediate their effects by binding to chemokine receptors found on the migrating cell's surface. Chemokine binding to the chemokine receptor results in signaling, which allows the cell to migrate towards the epicenter of chemokine production.

In addition to 'classical' chemokine receptors which are involved in leukocyte migration, a discrete family of chemokine receptors exist which are considered to be 'atypical', as binding to their cognate ligands does not result in classical signaling as detected by calcium flux assays. One of these atypical chemokine receptors is the chemokine-scavenging receptor D6, which can bind to and internalize at least 14 inflammatory CC chemokines *in vitro*. In addition, an analysis of D6 function *in vivo* has shown that D6 is important for the resolution of the inflammatory response. D6 KO mice treated with phorbol ester to the shaved dorsal skin developed an inflammatory skin pathology that resembled the human condition psoriasis in many respects. In contrast, WT mice treated with phorbol ester developed a very mild inflammatory response, which quickly resolved. These data suggested that a loss of D6 expression 'primed' the mouse to develop a psoriasisform pathology, requiring only minor irritation/trauma to develop the pathology. Similarly, histologically normal (uninvolved) skin from a psoriatic patient has a propensity to developing inflammatory lesions upon minor trauma, and could also be suggested to be 'primed' for lesion development. Collectively, these data led us to the following hypothesis,

'A loss of D6 expression in uninvolved psoriatic skin is associated with the development of psoriatic lesions'.

To test this hypothesis, D6 expression in clinical samples from psoriasis patients was analysed. Full thickness biopsies from psoriasis patients were taken from a histologically normal site (uninvolved psoriatic skin), in addition to an elliptical biopsy covering the skin directly adjacent to the psoriatic lesion (peri-lesional psoriatic skin), in addition to the lesion itself (lesional psoriatic skin). D6 expression was analysed in these biopsies by QPCR and immuno-staining. It was observed that D6 expression was significantly elevated in psoriatic skin compared to healthy control skin. In particular, in uninvolved psoriatic skin D6 was significantly increased compared to healthy control skin, or peri-lesional

psoriatic skin or lesional psoriatic skin. The increase in D6 expression in uninvolved psoriatic skin localised to the epidermis and the LVs. A significant increase in PBMC-D6 expression was also noted in psoriatic patients compared to healthy control PBMCs. These data suggest that at sites not directly involved in the pathology, D6 is elevated in an attempt to limit inflammation-induced damage.

Further immuno-staining showed the inflammatory CC chemokines CCL2 and CCL5 (both high affinity D6-binding ligands) were detected in uninvolved psoriatic epidermis, but were apparently unable to mediate their function due to the lack of significant leukocyte infiltration into the tissue. These data gave rise to the idea that D6 in uninvolved psoriatic skin was significantly elevated in an attempt to block the release of inflammatory CC chemokines into the dermis, and subsequent migration of inflammatory leukocytes into the tissue, and the onset of lesion formation. Interestingly, D6 expression on the epidermis was strongest towards the lower layers of the epidermis, which suggested a role for epidermal-D6 in 'barrier function', preventing the uncontrolled release of inflammatory CC chemokines into the dermis.

In addition to inflammatory CC chemokines, a variety of inflammatory cytokines have been previously detected in uninvolved psoriatic skin. Several of these cytokines were shown to increase D6 expression *in vitro* in this study. Therefore, it is possible the significant increase in D6 expression in uninvolved psoriatic skin is partly mediated by cytokine stimulation.

A loss of D6 expression was observed when comparing uninvolved psoriatic skin and peri-lesional psoriatic skin. These data suggested that a loss of D6 expression occurs directly before the onset of lesion formation. It was also shown in this study that a loss of D6 expression could occur after micro-trauma to uninvolved psoriatic skin, which suggests a possible mechanism of how D6 expression is lost in peri-lesional psoriatic skin.

To analyze whether the increase in D6 expression in psoriatic skin was disease specific, or a generic response to cutaneous inflammation, D6 expression in eczema skin was studied. It was found that D6 expression in eczema skin is elevated compared to healthy control skin, but less so compared to psoriatic skin. Collectively these data suggest that increased D6 expression may be a feature of inflammatory skin diseases.

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## List of publications arising from this work

- MCKIMMIE, C. S., **SINGH, M. D.**, HEWIT, K., LOPEZ-FRANCO, O., LE BROcq, M., ROSE-JOHN, S., LEE, K. M., BAKER, A. H., WHEAT, R., BLACKBOURN, D. J., NIBBS, R. J. & GRAHAM, G. J. 2013. An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood*.
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## **Author's Declaration**

The work presented in this thesis represents original work carried out by the author. This thesis has not been submitted in any form to any other university.

## Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AMPs	Antimicrobial peptides
APC	Antigen Presenting Cell
BDCA-1	Blood Dendritic Cell Antigen -1 protein
BDCA-2	Blood Dendritic Cell Antigen -2 protein
BM	Bone Marrow
cDNA	complimentary Deoxyribose Nucleic Acid
CFA	Complete Freund's Adjuvant
CFA	Complete Freund's Adjuvant
COPD	Chronic Obstructive Pulmonary Disease
Ct	Cycle Threshold
Cy5	Cyanine 5
DAB	3,3'-Diaminobenzidine
DAPI	4',6 – diamidino-2-phenylindole
DARC	Duffy Antigen Receptor for Chemokines
DMBA	7,12-dimethylbenz( <i>a</i> )anthracene
DNA	Deoxyribonucleic Acid
DSS	Dextran Sulphate Sodium
EAE	Experimental Autoimmune Encephalomyelitis
EEA1	Early Endosomal marker A1
FACS	Fluorescence Activated Cell Sorting
Fc	Fc receptor
FCS	Foetal Calf Serum
Fc $\epsilon$ RI	Fc epsilon receptor I
FITC	Fluorescein isothiocyanate
FLS	Fibroblast-like synoviocytes
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GFP	Green Fluorescence Protein
GWAS	Genome Wide Association Studies
H&E	Hematoxylin and eosin
HBD-2	Human Beta Defensin-2
HDLECs	Human Dermal Lymphatic Endothelial Cells
HEK	Human Embryonic Kidney

HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HLA	Human Leukocyte Antigen
iDC	Immature Dendritic Cell
IDECs	Inflammatory Dendritic Epidermal Cells
IF	Immunofluorescence
IFN $\alpha$	Interferon alpha
IFN $\beta$	Interferon beta
IFN $\gamma$	Interferon gamma
IgG	Immunoglobulin
IL-10	Interleukin-10
IL-12	Interleukin 12
IL-13	Interleukin-13
IL-15	Interleukin 15
IL-17	Interleukin 17
IL-1RA	Interleukin 1 receptor antagonist
IL-1 $\alpha$	Interleukin 1 alpha
IL-1 $\beta$	Interleukin 1 beta
IL-1 $\beta$	Interleukin 1 beta
IL-2	Interleukin 2
IL-21	Interleukin 21
IL-23	Interleukin 23
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin 6
IL-7	Interleukin 7
iNOS	inducible Nitric Oxide Synthase
K15	Keratin 15
K4	Keratin 4
kDA	kilodalton
KO	Knockout
LC	Langerhans Cells
LCM	Laser Capture Microscopy
LL-37	Human cathelicidin
LV	Lymphatic Vessel
mDC	Myeloid Dendritic Cell

MIP-1 $\alpha$	Macrophage Inflammatory Protein-1 alpha
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
Mtb	Mycobacterium tuberculosis
N	Number of samples
NHEKs	Normal Human Epidermal Keratinocytes
NK	Natural Killer
NOD	Non Obese Diabetic
nTreg	natural T-regulatory cell
PASI	Psoriasis Area Severity Index
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PsA	Psoriatic Arthritis
PSORS1	Psoriasis susceptibility loci
QPCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RNAse	Ribonuclease
SDF	Stromal cell Derived Factor 1
sIL-6R	Soluble IL-6 receptor
SNP	Single Nucleotide Polymorphism
SCC	Squamous Cell Carcinoma
SSC	Systemic Sclerosis
T-trophic	T-cell trophic
TAM	Tumour Associated Macrophages
TBP	TATA Binding Protein
TGF $\beta$	Transforming Growth Factor beta
Th1	Type 1 helper T-cell
Th2	Type 2 helper T-cell
Th22	Type 22 helper T-cell
Tip-DCs	Tumor Necrosis Factor and inducible nitric oxide synthase producing DCs
TLR7	Toll Like Receptor 7
TLR9	Toll Like Receptor 9
TNF $\alpha$	Tumour Necrosis Factor alpha

TPA	12-O-tetradecanoylphorbol-13-acetate
WT	Wild Type

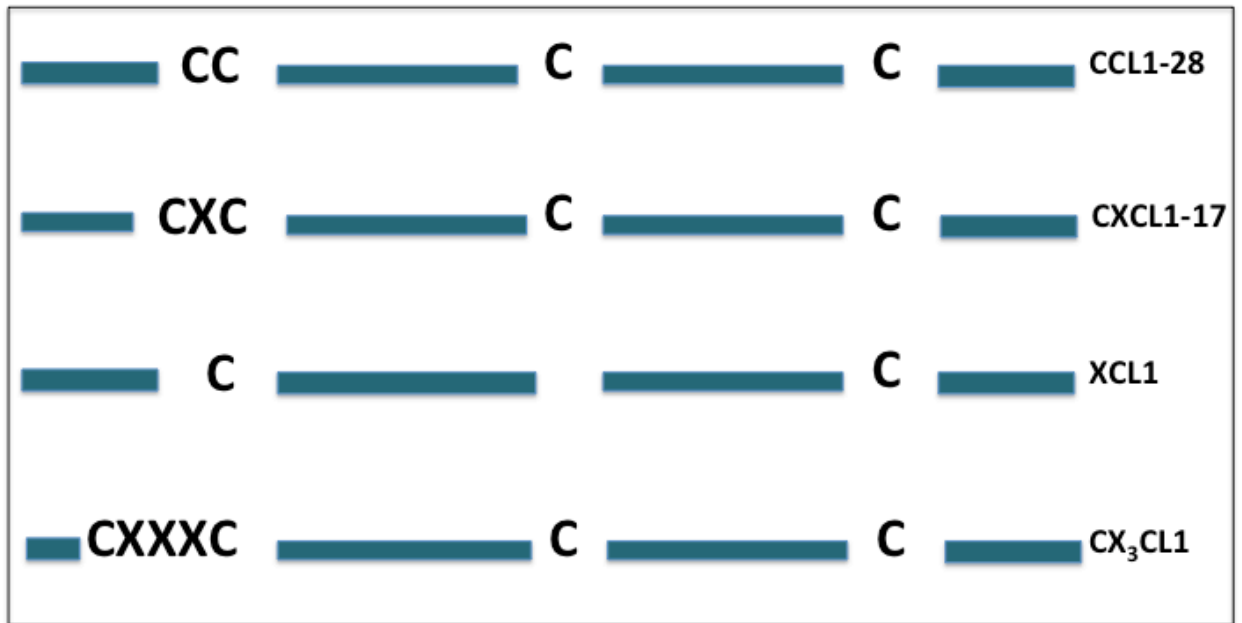
## **Chapter 1. Introduction**

# Introduction to chemokines and chemokine receptors

## 1.1. Chemokines

Chemokines are members of a family of evolutionarily conserved proteins approximately 6-10kDa in size, that are classified according to the presence of a conserved cysteine motif found within their mature sequence (Rot and von Andrian, 2004). Since their initial identification, over 20 years ago, the chemokine family has expanded and now contains more than 50 members (Nomiya et al., 2011). The most established function of chemokines is to direct the migration of leukocytes during inflammation and homeostasis (Zlotnik and Yoshie, 2000). Within the chemokine family, there are 4 subfamilies that contain variations of the cysteine motif (Table 1). The two largest subfamilies of chemokines are the CC chemokines and CXC chemokines. In addition there are XC and CX<sub>3</sub>C subfamilies that both contain single members (Rot and von Andrian, 2004). In addition to sub-categorising chemokines on the basis of variations in their cysteine motifs (See sections 1.1.1 – 1.1.3.), chemokines can also be broadly sub-divided into one of two categories, either inflammatory, or homeostatic, depending on their function *in vivo* (Zlotnik and Yoshie, 2000). Chemokines that are induced as a result of pathogenic insult are classified as inflammatory chemokines, whereas chemokines involved in basal leukocyte trafficking under steady state conditions are referred to as homeostatic chemokines (Zlotnik and Yoshie, 2000). Chemokines and their receptors in inflammation and homeostasis will be discussed in sections 1.3. and 1.4.

Possibly the earliest chemokine to evolve was CXCL12, which binds to CXCR4 (in addition to CXCR7, see section 1.12.). A series of elegant papers identified a prominent role for the binding of CXCL12 to CXCR4 in the development of the embryo (Ara et al., 2003, Molyneaux et al., 2003b, Knaut et al., 2003, Zou et al., 1998, Tachibana et al., 1998). Genetic deletion of CXCR4 was found to result in the death of the mice in utero (Zou et al., 1998, Tachibana et al., 1998). The lethal phenotype mirrored the phenotype observed upon genetic deletion of CXCL12 (Nagasawa et al., 1996). These data suggested that the interaction of CXCL12 with CXCR4 is essential for the proper development of the embryo. In particular, defects in cardiac development, in addition to haematopoiesis were observed (Nagasawa et al., 1996, Tachibana et al., 1998, Zou et al., 1998). These findings were extended by data from a number of papers which showed an essential, non redundant, role for CXCR4 migration towards CXCL12 in primordial germ cell migration within the



**Table 1.1. Variations in the conserved cysteine motif in the four chemokine subfamilies.**



developing embryo (Molyneaux et al., 2003a, Knaut et al., 2003, Ara et al., 2003, Doitsidou et al., 2002).

#### **1.1.1. CC Chemokines**

The CC chemokines are the largest sub-family of chemokines, comprising 28 members (CCL1 – CCL28) (Zlotnik and Yoshie, 2000, Rot and von Andrian, 2004). CC chemokines have four cysteine residues in their mature sequence, with the first two cysteines being adjacent to each other (hence ‘CC’). Chemokines from the CC sub-family are involved in both homeostatic and inflammatory processes (Zlotnik and Yoshie, 2000, Rot and von Andrian, 2004) (See sections 1.3 and 1.4). For example, CCL2 – CCL5 have established roles in attracting immature DCs (iDCs), monocytes and activated T-cells to sites of inflammation (e.g. in the skin). CCL17 and CCL22 are known to attract Th2 T-cells to sites of inflammation. CCL19 and CCL21 are homeostatic chemokines involved in coordinating the migration of T-cells and antigen presenting cells to lymph nodes (See section 1.4. for more detail).

#### **1.1.2. CXC Chemokines**

The CXC chemokine sub-family consists of 17 chemokines, all of which have four cysteine residues with a variable amino acid (i.e. ‘X’) between the first two cysteines (Zlotnik and Yoshie, 2000, Rot and von Andrian, 2004). Similar to CC chemokines, CXC chemokines have roles both in homeostatic, and inflammatory contexts. The most well known CXC chemokine is CXCL8, which was formerly believed to be a cytokine (initially called IL-8). CXCL8 has a major role in attracting polymorphonuclear cells to sites of inflammation, as it is a high affinity chemoattractant for neutrophils (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). CXCL9, CXCL10 and CXCL11 are important in the context of recruiting activated Th1 T-cells to sites of inflammation in a variety of diseases, including psoriasis (See section 1.6.17.). Similar to CC chemokines, some CXC chemokines are involved in controlling homeostatic movement of leukocytes. For example, CXCL13 has an established role in B-cell positioning in the lymph node (See section 1.4.). CXCL16 is one of two chemokines which has a transmembrane domain (the other CX3CL1 is discussed in section 1.1.3.), and can be either membrane bound, or soluble in form (Matloubian et al., 2000, Wilbanks et al., 2001). CXCL16 is expressed on DCs, in addition to monocytes, macrophages and B-cells (Matloubian et al., 2000, Wilbanks et al., 2001, La Porta, 2012), and is thought to have a role in tumour progression (La Porta, 2012).

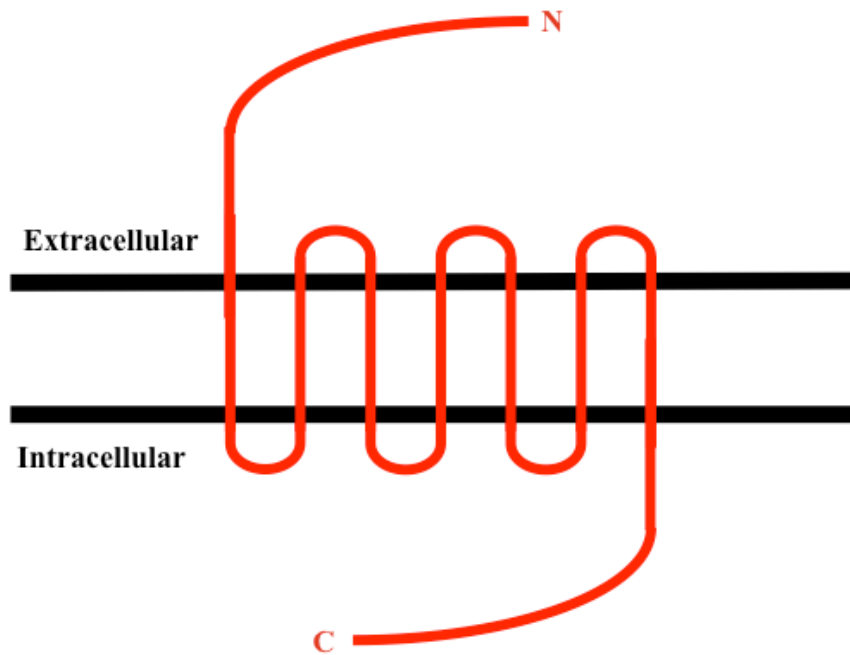
### **1.1.3. XC and CX3C Chemokines**

In addition to the larger chemokine sub-families (CC and CXC), there are two additional chemokine sub-families, each with a single member (Zlotnik and Yoshie, 2000, Rot and von Andrian, 2004). CX3CL1 was identified by a group looking for novel chemokine sequences (Bazan et al., 1997). CX3CL1 expression was found in a variety of tissues, including the colon, prostate, heart and skeletal muscle (Bazan et al., 1997). Weak, but detectable expression of CX3CL1 was also detected in peripheral blood leukocytes (PBLs) (Bazan et al., 1997). This study demonstrated that CX3CL1 was a transmembrane protein, with the chemokine protruding from the cell membrane, attached to the C-terminal (Bazan et al., 1997). In addition, this study showed that chemokine could be released from the stalk, which implied alternative biological functions for the soluble, and membrane bound, forms of CX3CL1 (Bazan et al., 1997). The soluble form was found to be chemoattractive for a variety of cell populations, including T-cells, monocytes, NK cells, and B-cells (Bazan et al., 1997, Imai et al., 1997). Of notable interest was that CX3CL1 was found to be more potently chemoattractive for monocytes and T-cells compared to CCL5, suggesting that CX3CL1 may have a prominent role in the chemotaxis of these cell types (Bazan et al., 1997). Schall and colleagues also provided evidence that the membrane bound form of CX3CL1 could function as an adhesion molecule for T-cells and monocytes (Bazan et al., 1997).

XCL1 is the sole member of the fourth sub-family of chemokines. It was identified during screening of cDNA libraries generated from pro-T cells (T-cells in the final differentiation stages) looking for new cytokines (Kelner et al., 1994). XCL1 is expressed by pro-T-cells, CD8<sup>+</sup> T-cells,  $\gamma\delta$  T-cells, NK cells, NK-T cells, and thymic medullary epithelial cells (Dorner et al., 2002, Dorner et al., 2004, Boismenu et al., 1996, Lei et al., 2011).

## **1.2. Chemokine receptors**

Chemokines mediate their effects by binding to chemokine receptors (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). Chemokine receptors belong to the 7-transmembrane spanning family of G-protein coupled receptors (GPCRs) (Figure 1.1.) (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000, Murphy et al., 2000). Ligation of chemokines to their receptors leads to signal transduction and subsequent downstream effects. Chemokine receptors can be sub-divided into four sub-families on the basis of the chemokines they bind (Table 2.). (Zlotnik and Yoshie, 2000).



**Figure 1.1. Illustration of a chemokine receptor**

All chemokine receptors are seven transmembrane spanning G-protein coupled receptors (GPCRs). The N-terminus binds to the chemokines, and the C-terminus couples the receptor to G-proteins, which mediates signaling.

Receptor	Ligands
CCR1	CCL3, 3L1, 5, 7, 8, 13, 14, 15, 16, 23
CCR2	CCL2, 7, 8, 13, 16
CCR3	CCL5, 7, 8, 11, 13, 15, 24, 26, 28
CCR4	CCL17, 22
CCR5	CCL3, 3L1, 4, 4L1, 5, 8
CCR6	CCL20
CCR7	CCL19, 21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27, 28
CXCR1	CXCL6, 8
CXCR2	CXCL1, 2, 3, 5, 6, 7, 8,
CXCR3	CXCL9, 10, 11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CX3CR1	CX3CL1
XCR	CXCL1, 2

**Table 1.2. Members of the four chemokine receptor sub-families and their respective ligands.**

The above table lists all the chemokine receptors and their ligands, and is colour coded according to sub-family. I.e. CC chemokine receptors/ligands in blue, CXC chemokine receptors/ligands in red, CX3C chemokine receptor/ligand in green, XCR chemokine receptor/ligand in yellow.

### **1.2.1. CC Chemokine receptors**

There are 10 chemokine receptors that bind CC chemokines (Table 2.). Similar to their ligands, CC chemokine receptors are thought of as either inflammatory, or homeostatic depending on the contexts in which they function. CCR1-3, and CCR5 are considered to be inflammatory CC chemokine receptors (Rot and von Andrian, 2004, Murphy et al., 2000). CCR2 is implicated in the migration of monocytes to sites of inflammation, although CCR1 and CCR5 have also been reported to attract monocytes to areas of inflammation (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000, Murphy et al., 2000). CCR2 is also known to have a role in the egress of monocytes from the bone marrow (Tsou et al., 2007). CCR5 is also known to recruit CD3+T-cells to the skin in the context of Psoriasis (de Groot et al., 2007). CCR7, CCR9 and CCR10 are examples of chemokine receptors that are involved in homeostatic functions (Zlotnik and Yoshie, 2000, Murphy et al., 2000, Rot and von Andrian, 2004). CCR7 is involved in T-cell/DC migration to the lymph nodes (See section 1.4.). CCR9 is involved in T-cell migration to the gut (Koenecke and Forster, 2009). CCR10 has two ligands, CCL27 and CCL28. Under homeostatic conditions, T-cells migrate to the skin via CCR10 mediated chemotaxis (Zlotnik and Yoshie, 2000, Murphy et al., 2000, Rot and von Andrian, 2004). However, under inflammatory conditions such as psoriasis, this can result in elevated T-cell accumulation in the skin (See section 1.6.17.).

CCR6 has only 1 ligand, CCL20. This monogamous chemokine – chemokine receptor interaction has been shown to be essential for the migration of Th17 cells into the central nervous system in Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of brain inflammation (Reboldi et al., 2009). CCR3 is known to attract eosinophils in the context of eczema (see section 1.7.7.). Th2 cells are known to migrate to eczema skin via CCR4. CCR3 and CCR4 are associated with allergy-based inflammatory disorders. These are considered to be Th2 dominated diseases (Luster, 2001).

### **1.2.2. CXC Chemokine receptors**

There are 6 chemokine receptors that bind CXC chemokines (CXCR1-CXCR6) (Table 2.) (Zlotnik and Yoshie, 2000, Murphy et al., 2000, Rot and von Andrian, 2004). Similar to CC chemokine receptors, they can be classified according to the context in which they function, either inflammatory (CXCR1-3), or homeostatic (CXCR4, CXCR5 and CXCR6). CXCR1 and CXCR2 are expressed on neutrophils, and their best-understood role is in the regulation of migration of neutrophils to sites of inflammation (Zlotnik and Yoshie, 2000, Murphy et al., 2000, Rot and von Andrian, 2004). CXCR3 is considered to be one of the chemokine receptors most associated with Th1 cells, the other being CCR5 (Campbell and

HayGlass, 2000). CXCR3 mediates T-cell migration to inflammatory sites (See section 1.16.17.) (Groom and Luster, 2011a, Groom and Luster, 2011b). CXCR4 has 1 ligand, CXCL12 (Murphy et al., 2000, Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). The interaction of CXCR4 and CXCL12 in the context of development has already been discussed (Section 1.1.). In addition, CXCR4 has a non-redundant role in maintaining hematopoietic stem cells (HSCs) within the bone marrow (Aiuti et al., 1997, D'Apuzzo et al., 1997, Murphy et al., 2000, Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). CXCR5 has a role in the homing of B-cells to the B-cell follicle in the LN, and will be discussed in section 1.4.. CXCR6 also has only one ligand, CXCL16. The interaction with CXCR6 and its ligand is believed to have a minor role in the entry of HIV to T lymphocytes (Matloubian et al., 2000), in addition to cancer as both CXCR6, and its ligand are over-expressed in both cancer tissue and cancer cell lines (La Porta, 2012).

### **1.2.3. XC and CX3C Chemokine receptors**

XCR1 is the only member of the XC family of chemokine receptors (Table 2.). XCR1 was initially thought to be expressed on a variety of cell types (Lei and Takahama, 2012). However, subsequent studies have identified that XCR1 is expressed exclusively on a specific sub population of DCs (Dorner et al., 2009). Specifically, XCR1 was reported to be expressed on CD8<sup>+</sup> mouse DCs (Dorner et al., 2009). Dalod and colleagues expanded upon these findings by confirming that XCR1 is expressed on CD8<sup>+</sup> mouse DCs, in addition to showing that homologous populations of DCs exist in humans, and sheep (Croizat et al., 2010). Therefore, XCR1 was suggested to be a potential marker of a sub-population of DCs (Croizat et al., 2010).

CX3CR1 is the sole member of the CX3C chemokine receptors (Table 2.), and is expressed on a variety of cell types, including lymphocytes and endothelial cells (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000, Murphy et al., 2000). The receptor for CX3CL1 (Imai et al., 1997), was identified shortly after the publication of CX3CL1 (Bazan et al., 1997). The orphan receptor V28 (referred to as CX3CR1 from this point on), previously identified as a GPCR (Combadiere et al., 1995, Raport et al., 1995), was found to be the receptor for CX3CL1 (Imai et al., 1997). CX3CR1 expression was found on NK cells T-cells and monocytes (Imai et al., 1997). Very weak, but detectable expression was also identified on B-cells (Imai et al., 1997, Raport et al., 1995). The *in vivo* function of CX3CR1 was further delineated by Patel and colleagues, who showed that membrane bound CX3CL1 can mediate the adhesion of leukocytes through binding to CX3CR1 under physiologically relevant shear force stresses (Fong et al., 1998).

### 1.3. In inflammation

Inflammatory chemokines can be produced by almost any cell in the body in order to direct the migration of leukocytes to specific sites during an inflammatory response (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). Under non-inflammatory conditions, inflammatory chemokines are generally not detectable. Upon infection or damage to the tissue, inflammatory chemokine production is quickly induced, in order to facilitate the appropriate leukocyte recruitment to the site of inflammation (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). The production of inflammatory chemokines occurs soon after tissue damage or pathogen insult, and the production of inflammatory chemokines is generally transient (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). This transient production is an important feature of these chemokines. If inflammatory chemokine production is prolonged, it can lead to effects that are deleterious to the host, and precipitate chronic inflammatory diseases such as psoriasis (see section 1.6.). CCLs1-5 and CXCLs1-11 are examples of inflammatory chemokines (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). It is generally believed that CXCL1-8 are involved in neutrophil migration to sites of inflammation, and CXCL9-11 are the primary mediators of activated T-cell migration. Inflammatory CC chemokines, such as CCL1-5 are predominantly involved in the migration of monocytes and T-cells to the sites of inflammation (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). However, there are other chemokines that have known roles in leukocyte migration to sites of inflammation. Examples of these include, CCL27, CCL28, CCL20, CCL17 and CCL22. The role of these chemokines and their receptors are discussed within the context of psoriasis and eczema in sections 1.6.17. and 1.7.7.

T-cells migrate into peripheral tissues through CXCL9-11 induced chemotaxis via CXCR3 found on T-cells (Groom and Luster, 2011a, Groom and Luster, 2011b). However it should be noted here that T-cells express other chemokine receptors on their surface such as CCR5 and CXCR2. The ligands for CCR5 (e.g. CCLs3-5) and CXCR2 (CXCLs1, 2 and 8) can also induce T-cell migration to sites of inflammation. In addition, both myeloid and plasmacytoid dendritic cells (DCs) will migrate towards both CXC and CC chemokines (Penna et al., 2002). In addition, neutrophils express CCR1 (Lionakis et al., 2012), and are therefore responsive to CCL3 and CCL5. As can be seen from the examples above, it is clear that inflammatory chemokines and their respective receptors have a great deal of promiscuity in terms of their binding profiles (Mantovani, 1999). Upon genetic deletion of any one inflammatory chemokine or chemokine receptor, in general, there is no major

effect on the resting phenotype, or during inflammation. In CCL5 KO mice and CCL11 KO mice, there were no obvious histological or developmental defects between WT mice and KO mice (Makino et al., 2002, Rothenberg et al., 1997). Admittedly, a modest reduction in the number of CD4+memory T-cells in the spleen of CCL5 KO mice, and a reduction in the numbers of circulating eosinophils in CCL11 KO mice was observed compared to WT mice (Makino et al., 2002, Rothenberg et al., 1997). However, these KO mice were otherwise unaffected which shows that a loss of an inflammatory chemokine results in only minor changes to the animals physiology. These data suggest that there are other chemokine-chemokine receptor interactions, which can compensate for the loss of one chemokine-chemokine receptor interaction.

This has led to the description of chemokines and their receptors involved in inflammation as being functionally redundant (Mantovani, 1999). The first inflammatory CC chemokine to be genetically deleted was CCL3 (Cook et al., 1995). No phenotype was observed in the resting state of CCL3 KO mice compared to WT mice (Cook et al., 1995). Upon infection with influenza virus, CCL3 KO mice cleared the infection, although this was delayed compared with WT mice. However, the virus was ultimately cleared (Cook et al., 1995). Therefore other inflammatory chemokines or cytokines were most likely involved in clearing the infection.

#### **1.4. In homeostasis**

In stark contrast to inflammatory chemokines, the expression of homeostatic chemokines is strictly controlled, and is a cell specific and tissue specific phenomenon (Rot and von Andrian, 2004, Zlotnik and Yoshie, 2000). Homeostatic chemokines are involved in basal leukocyte trafficking to various parts of the body. For example, CCL19 and CCL21 ensure efficient trafficking of DCs and T-cells to the lymph nodes. (Forster et al., 2008, Moschovakis and Forster, 2012). In CCR7 KO mice, lymph nodes contained virtually no T-cells (Forster et al., 1999). This was attributed to a lack of CCR7 on the T-cells preventing migration to the CCL19/CCL21 rich areas (T-cell zones) of the lymph node (Forster et al., 1999). A lack of CCR7 also affects the lymph node homing capabilities of DCs (Forster et al., 1999). T-cells in the dermis of CCR7 KO mice stimulated by painting the skin with fluorescein isothiocyanate (FITC) were unable to leave the dermis and move to the lymph nodes (Forster et al., 1999). In addition, a loss of CCR7 significantly reduced T-cell motility in the lymph nodes (Worbs et al., 2007). Thus CCR7 has a role in the speed of cell movement (Worbs et al., 2007). Therefore, the interaction between CCR7 and

CCL19/21 is clearly important for efficient lymph node homing of certain subsets of leukocytes. However, it was noted in CCR7 KO mice that the number of B-cells found in the lymph node were unaffected, although they are also known to express CCR7 (Forster et al., 1999). The lymph node homing capability of B-cells has been attributed to the interaction of CXCR5 on B-cells (Forster et al., 1996) and CXCL13 found in the B-cell area of the lymph node (Gunn et al., 1998, Legler et al., 1998). Interestingly, upon activation of B-cells in the follicle, B-cells down-regulate CXCR5, and up-regulate CCR7 (Reif et al., 2002). Similarly, activated T-cells up-regulate CXCR5 concomitant with a reduction in CCR7 expression (Hardtke et al., 2005). Therefore, activation of the B-cells and T-cells, and subsequent chemokine receptor switching, allows them to migrate closer to each other, thus allowing T-cells, and B-cells to interact with each other.

Other examples of homeostatic chemokine function include the migration of CCR9 expressing T-cells to the gut via an interaction with CCL25, and the migration of CCR10+ T-cells to the skin via CCL27 and CCL28 (Youn et al., 1999, Zaballos et al., 1999). Although the interaction of CCR7, CCR9 and CCR10 with their respective ligands has known roles in homeostasis, all three interactions have been shown to be involved in inflammatory contexts as well (Rot and von Andrian, 2004). In Crohn's disease, aberrant accumulation of T-cells in the gut occurs partially due to the migration of CCR9+ T-cells towards CCL25 in the gut (Papadakis et al., 2001). Similarly, T-cell accumulation in skin disorders such as psoriasis due to the migration of CCR10 positive T-cells has been reported (see section 1.6.). Also, elevated CCR7 and CCL19 and CCL21 have also been reported to be involved in the formation of dermal aggregates resembling lymphoid structures in psoriasis (See section 1.6.).

## **1.5. Chemokine receptors in disease**

Due to the promiscuity of chemokine and chemokine receptor binding, and their established roles in the trafficking of leukocytes around the body, it is not surprising that chemokines have been implicated in a large number of human diseases. It is not possible to discuss every human disease chemokines are implicated in (keying in 'chemokine and disease' to PubMed brings up more than 19,000 articles). Therefore, a select number of diseases with central chemokine involvement in their pathogenesis will be summarized.



### **1.5.1. Cancer**

One of the potential outcomes of unchecked cell proliferation, is the transformation of normal cells into cancerous cells, and subsequent tumour formation (Balkwill, 2004). The initial tumour (primary tumour as its often referred to), can move to another location, and set up secondary tumours. This process is referred to as metastasis (Viola et al., 2012). For primary tumours to move to secondary locations effectively, the primary tumour must use the bodies natural conduit systems such as the LVs, and the vasculature. As chemokines are essential for leukocyte migration around the body, it is not surprising that tumours also make use of chemokines, and their receptors, to migrate to a secondary location (Balkwill, 2004, Balkwill, 2012). In addition to the role of chemokines and their receptors in metastasis, the chemokine system is known to affect the growth of the tumour, in addition to the tumour's survival (Viola et al., 2012).

#### **1.5.1.1. Metastasis in cancer**

There is a large body of evidence suggesting that chemokines are important in guiding the tumour to a secondary location during metastasis, and there are many examples of this. Metastasis is not a random process, but an ordered sequence of events, whereby the tumour will move to a secondary location, based (in some cases) on the chemokine receptor profile of the tumour cells (Muller et al., 2001). CXCR4 is found on a wide variety of cancer cells, and is probably the most studied chemokine receptor in the context of cancer (Balkwill, 2004, Balkwill, 2012). CXCR4 expression has been identified on more than 20 different types of cancer cells, including breast cancer, renal-cell carcinoma, and prostate cancer (Balkwill, 2004, Balkwill, 2012, Sun et al., 2003, Staller et al., 2003, Mehta et al., 2007). However, there are many other chemokine receptors found on cancer cells including CXCR1-3, CX3CR1, CCR3, CCR4, CCR7 and CCR9-10 (Balkwill, 2004, Balkwill, 2012). Therefore, it is not surprising that metastasis of cancer cells, and the formation of secondary tumours can occur at a number of locations in the body. Melanoma cells with metastatic potential tend to have strong expression of CCR10, which results in their homing to the skin, a site of CCL27 production (Muller et al., 2001). In addition, CCR3 and CCR4 expression on malignant cells have been shown to be involved in the migration of malignant T-cells to the skin (Kleinhans et al., 2003, Ishida et al., 2003). CCR7 expression on cells results in the metastasis of the cancer cells to the lymph nodes, and has been demonstrated in a number of different cancers, including breast cancer, gastric cancer, and oesophageal cancer (Muller et al., 2001, Mashino et al., 2002, Ding et al., 2003). In addition, CCR7 expression on cancer cells has been associated with poor prognosis, and correlates with the cancer cell's metastatic potential (Balkwill, 2004).

#### **1.5.1.2. Inflammation in cancer**

In addition to cancer cells, tumours often have large numbers of leukocytes within them, particularly macrophages, and lymphocytes (Mantovani et al., 2008). Macrophages are found in tumours, and are termed ‘Tumour associated macrophages’ (Mantovani et al., 2008). CCL2 and CCL5 are established in monocyte/macrophage migration, and the levels of these CC chemokines correlate with tumour infiltration of macrophages and myeloid cells (Allavena et al., 2011, Mantovani et al., 2008). Tumour associated macrophages (TAMs) are known to enhance tumour growth, and TAM counts inversely correlate with patient prognosis (Allavena et al., 2011, Mantovani et al., 2008). Therefore, elevated production of CCL2 and CCL5 is actually of benefit to the tumour, allowing further infiltration of the tumour by macrophages. Uncontrolled inflammatory CC chemokine production has been shown directly to enhance tumour burden (see section 1.9.4.3.).

Lymphocytes, similar to TAMs, are recruited to the cancer via CC chemokines, in addition to CXC chemokines. (De Monte et al., 2011) A recent study where Th2 cells were found to enhance tumour growth was published by Protti and colleagues (De Monte et al., 2011). This study observed that a high Th2 cell counts resulted in a poor prognosis (De Monte et al., 2011).

Therefore within the context of cancer biology, chemokines, both homeostatic and inflammatory, can have a detrimental effect on the ability of the immune system to destroy the cancerous cells. From the brief discussion above, it would appear that attempting to target chemokines and/or their receptors, particularly in the case of CCL2 and CCL5, may be of some therapeutic benefit.

#### **1.5.2. Human immunodeficiency virus (HIV) infection**

HIV is a retrovirus first identified in 1983, and it kills infected individuals by targeting their immune system and destroying it (Gallo and Montagnier, 2003). Infection with HIV ultimately leads to the infected individual developing acquired immunodeficiency syndrome (AIDS), which then leads to death (Gallo and Montagnier, 2003). At present there is no cure for HIV. HIV infects and destroys macrophages and monocytes, in addition to the CD4+ T-cells. Thus rendering the infected individual very susceptible to a wide variety of infections and/or cancers. The virus gains entry to the cell using the CD4 protein on the cells, in addition to two co-receptors, the chemokine receptors CCR5, and CXCR4 (Kalinkovich et al., 1999, Broder and Collman, 1997). The macrophage tropic (M-

tropic) stage of HIV infection occurs first, and the co-receptor CCR5 allows the virus to infect macrophages and monocytes, in addition to CCR5+ T-cells (predominantly Th1 T-cells). Once the initial HIV infection has occurred, the virus stays in the infected cells, and starts to replicate (Kalinkovich et al., 1999, Broder and Collman, 1997).

The T-cell tropic (T-tropic) stage of the disease, occurs once the virus acquires the capability to infect CD4+T-cells using the chemokine receptor CXCR4. This leads to a significant increase in the virus' replication and infective potential, resulting in a drastic reduction in the infected individual's CD4+T-cell count (Kalinkovich et al., 1999, Broder and Collman, 1997). This reduction in CD4+T-cells allows opportunistic infections to take hold, and the onset of AIDs then ensues. The change of chemokine receptor used to infect the cells, from CCR5 to CXCR4, is an important stage that results in the infected individual developing AIDS.

Once it became known that chemokine receptors were essential for viral entry into the cell, studies were undertaken looking at the potential of chemokines to block virus mediated entry. The ligands for CCR5 (i.e. CCL3-CCL5) have been shown to inhibit HIV entry *in vitro* (Cocchi et al., 1995, Nibbs et al., 1999). In addition, infected individuals with high levels of CCL5 were found to be resistant to disease progression to AIDS (Clerici et al., 1996). As interaction of both chemokines, or HIV, leads to down-regulation of the chemokine receptor, studies were undertaken to analyse whether individuals that were deficient in CCR5 had a survival advantage over those that were CCR5 sufficient. It was subsequently discovered there were individuals infected with HIV homozygous for CCR5- $\Delta$ 32 (results in a truncated CCR5 protein, and a loss of signaling via this receptor). CCR5- $\Delta$ 32 homozygous individuals have significantly greater protection from HIV infection compared to individuals who are not homozygous for CCR5- $\Delta$ 32 (Huang et al., 1996). Individuals that are heterozygous for CCR5- $\Delta$ 32 have no enhanced protection from infection, but the progression of the disease to AIDS is slower (Misrahi et al., 1998, Buseyne et al., 1998). This mutation results in elevated CCL3-CCL5 release from PBMCs. A negative correlation was observed between high CCL5 production, and low plasma viremia (Saha et al., 1998). I.e., the higher the levels of CCL5, the lower the viremia, and the lower the rate of infection.

No mutation in the CXCR4 sequence has been found which confers resistance to HIV infection. However, a mutated CXCL12 (called SDF1-3'A due to the mutation in the 3' untranslated region) was identified (Winkler et al., 1998). While this did not provide any

resistance to HIV infection, infected individuals who were homozygous for this mutation were found to be 'slow progressors' to AIDS. Heterozygosity for SDF1-3' was found to confer no advantage over the wild type alleles (Winkler et al., 1998).

Therefore, evidence suggested that the co-receptors for HIV (CCR5 and CXCR4) were potential therapeutic targets. In a seminal paper, it was shown that use of an anti-CCR5 (commercially known as Maraviroc) antibody to block the co-receptor for HIV, could significantly reduce the viral load in HIV infected individuals (Fatkenheuer et al., 2005). Therefore, a therapeutic specifically targeting a chemokine receptor, is now used to treat a prevalent human disease. However, great care must be taken when considering the potential usefulness of CCR5 blockers in treating patients as it is possible use of these blockers could increase the susceptibility of the individual to West Nile Virus (WNV) infection. It was shown by Murphy and colleagues that CCR5 KO mice infected with WNV all succumbed to the infection, thus showing a non-redundant role for CCR5 in clearing WNV infection (Glass et al., 2005). In a separate study from the same group, it was noted that CCR5-Δ32 homozygous individuals had a greater chance of developing symptomatic WNV infection (Glass et al., 2006).

### **1.5.3. Rheumatoid arthritis**

Rheumatoid arthritis (RA) is a chronic inflammatory condition, which affects approximately 1% of the world's population (McInnes and Schett, 2011). The pathogenesis of RA involves infiltration of the synovium of the joint by a variety of leukocytes, including T-cell, B-cells, Neutrophils, NK cells, DCs, and plasma cells (McInnes and Schett, 2011). The major symptoms of RA are the swelling of the joint synovium due to inflammation, which occurs symmetrically, and concurrently with stiffness, and discomfort for the patient (Iwamoto et al., 2008). As RA is a chronic inflammatory disorder, it is no surprise to find that chemokines are elevated in the RA synovium (Iwamoto et al., 2008). Neutrophil attracting chemokines CXCL1 and CXCL8 are notably expressed in the synovial tissue and synovial fluid (Koch et al., 1992, Koch et al., 1995). In addition, inflammatory cytokines found in the synovium, such as IL-17, TNFα, and IL-1β can induce production of these chemokines from resident synovial cells, including fibroblast-like synoviocytes (FLS), and articular chondrocytes. T-cell attracting chemokines, such as CXCL9 and CXCL10, in addition to CCL3 and CCL5 are also elevated in the synovium (Patel et al., 2001, Hanaoka et al., 2003). These T-cell chemoattractants, similar to the neutrophil attracting chemokines, can be elevated *in vitro* through stimulation of FLS and other cellular components of the synovium with inflammatory mediators, such as

cytokines. There are many more examples of chemokines found in the synovium, and these examples cover the four subfamilies of chemokines (Iwamoto et al., 2008).

Therefore, it appears that chemokines have an important role in recruiting the various leukocyte populations to the synovium. Interestingly, it appears that certain inflammatory chemokine receptors, and the interaction they have with their ligands, can confer some protection to RA patients. The CCR5 $\Delta$ 32 mutation discussed in the context of HIV, was found to negatively correlate with RA disease progression (Pralhad, 2006). Using animal models of RA, it was found that blocking CCR5 reduced the severity of the disease. These data suggested that CCR5 might be a potential therapeutic target in RA. Therefore, similar to HIV, CCR5 $\Delta$ 32 mutation confers resistance to disease progression. Overall, these data show that chemokines, and their receptors are involved in RA pathogenesis.

#### **1.5.4. Psoriatic arthritis**

Psoriatic arthritis (PsA), similar to RA is an inflammatory joint disease (Fitzgerald and Winchester, 2009). As a major characteristic of PsA and RA is swollen joints, it is not easy to distinguish between the diseases (Fitzgerald and Winchester, 2009). However, some differences have now been identified. In RA, there is a greater number of CD83<sup>+</sup> DCs, and the synovial membrane is thicker compared to PsA (Kruithof et al., 2005). On the other hand, there are greater numbers of macrophages and neutrophils in the PsA synovium compared to the RA synovium. In addition, there is more pronounced vascularity in PsA synovium when examined against RA synovium (Kruithof et al., 2005). Approximately 30-45% of patients with PsA also have psoriasis. The HLA-C allele Cw\*0602, which is a susceptibility allele for psoriasis, has also been identified as a PsA susceptibility allele (Fitzgerald and Winchester, 2009). Similar to psoriasis (albeit at different tissue sites), there is expression of inflammatory mediators in PsA synovium. In particular, IL-6, IL-12, IL-15 and IL-17, in addition to TNF $\alpha$  have been detected in PsA synovium (Pene et al., 2008, van Kuijk et al., 2006). These cytokines have all been detected in psoriasis lesions. In addition, homeostatic chemokines CCL21 and CXCL13 have been detected in both psoriasis lesions, and the PsA synovium (Canete et al., 2007). Until recently it has been difficult to carefully examine, and diagnose the full extent of the disease pathology in PsA patients. However, imaging technology developments are now making it possible to obtain more detail on the pathogenesis of PsA in patients, and this should enable clinicians and scientists to further characterize the disease (Fitzgerald and Winchester, 2009). It is possible that new developments from these studies may yield additional therapeutic targets for PsA.

### **1.5.5. Chemokines in cutaneous disease**

Skin inflammation involves the influx of a variety of inflammatory leukocyte populations into the skin. Cell populations involved in inflammatory skin diseases include T-cells (Th1, Th2, Th17 or Th22 depending on the disease), in addition to various populations of DCs (pDCs, mDCs, LCs), macrophages (both type 1 and type 2 macrophages), in addition to mast cells, and neutrophils and eosinophils (Homey and Bunemann, 2004, Pastore et al., 2004). With such a large variety of inflammatory cells involved in cutaneous inflammation, it is perhaps not surprising to note that a variety of different inflammatory chemokines are expressed within the skin during inflammation. Neutrophil infiltration of the skin tends to be mediated by CXCL2 and CXCL8 (Rot and von Andrian, 2004). T-cells in comparison, can be induced to enter the inflammatory skin by a multitude of inflammatory chemokines including CXCL9, CXCL10, CXCL11, CCL2, CCL4, CCL5, CCL13, CCL17, CCL22, CCL20 and CCL27 (Murphy et al., 2000, Rot and von Andrian, 2004). Macrophages and DCs are known to migrate into the skin via CCL2, CCL4, CCL5, in addition to CCL20 in the case of DCs (Murphy et al., 2000, Rot and von Andrian, 2004). Eosinophils respond to a multitude of inflammatory chemokines, although CCL11 has been most heavily implicated with eosinophil migration (Murphy et al., 2000, Rot and von Andrian, 2004).

Due to the number of different inflammatory chemokines involved, it can be difficult to look for patterns of inflammatory chemokine expression that suggest that one/several inflammatory chemokines might be important in the cellular influx during cutaneous inflammation. However, certain inflammatory chemokines are common to several different skin pathologies. These include CCL20 and CCL27, in addition to CXCL10 (Pastore et al., 2004). These chemokines will be discussed in the context of psoriasis and eczema in sections 1.6.17. and 1.7.7. However, certain chemokines are more restricted to specific cutaneous disorders, such as CCL1, CCL11 and CCL18, which are discussed in sections 1.7.6. and 1.7.7. Therefore, while certain inflammatory chemokines (e.g. CCL3, CCL4, CCL5 and CXCL10) can bring in a 'leukocyte army' that is relatively non-specific for the particular inflammatory skin disease, certain chemokines (particularly CCL11 and CCL18) tend to be associated with particular cutaneous diseases.

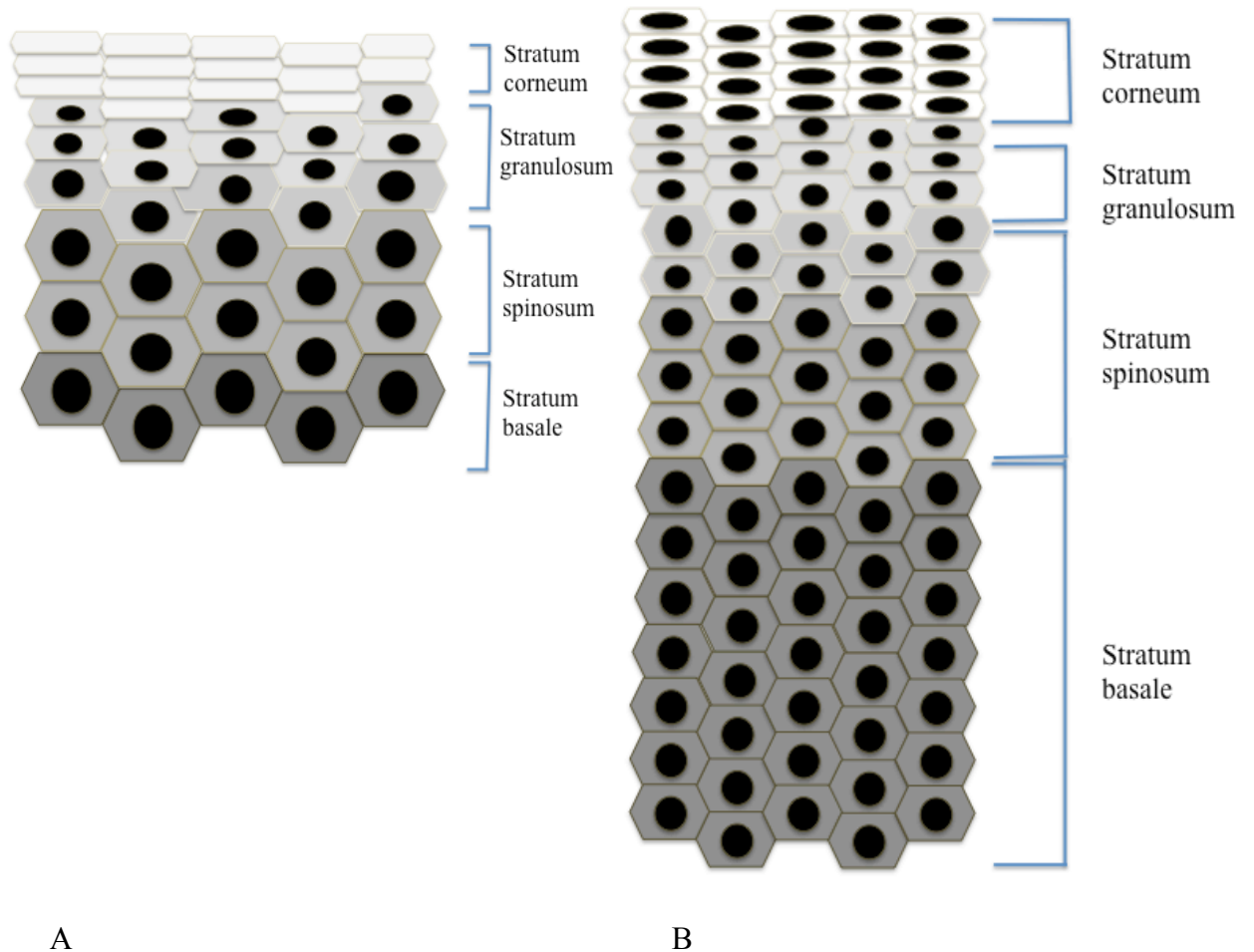
## 1.6. Psoriasis

### 1.6.1. Introduction

Psoriasis is a chronic inflammatory disease affecting approximately 2% of the population worldwide (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). There are several types of psoriasis although the predominant form is named psoriasis vulgaris (referred to as psoriasis from now on) and this accounts for more than 90% of all psoriasis cases. This is the form of psoriasis we will discuss in this section. On a macroscopic scale (clinical appearance), psoriasis patients have well demarcated skin lesions that are red with silvery/white scales (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). In addition these lesions are noticeably elevated compared with non-lesional skin due to thickening of the epidermis (acanthosis)

Using microscopy, psoriatic skin is markedly different from healthy skin. Psoriatic skin displays excessive epidermal proliferation (hyperplasia) (Figure 1.2.), an inhibition of the differentiation of the keratinocyte layers, in addition to large numbers of leukocytes infiltrating both the dermal and epidermal regions of the lesion (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). There is a variety of leukocyte populations found in psoriatic lesions including Dendritic cells (mDCs, pDCs, Langerhans cells), helper T-cell subsets (Th1, Th17, Th22), CD8+ T cells, neutrophils, mast cells and macrophages. The main leukocyte types found in the epidermis are CD8+ T cells, in addition to LCs and neutrophils. The dermal infiltrate is composed of helper T-cells, in addition to various DC subsets and mast cells and macrophages (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012).

Due to the social implications of this disfiguring disease, psoriasis patients tend to have a reduced quality of life when compared with their healthy counterparts, and have an increased risk of clinical depression in addition to death at a younger age (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012, Krueger et al., 2001). As patients with psoriasis are often on treatment for years, and in some case decades, it is clearly a major drain on society's resources. At present there is no cure for psoriasis. However, even if patients go into full remission the disease can re-appear, and therefore a greater understanding of the



**Figure 1.2. Epidermal structure in healthy skin and psoriasis lesional skin (adopted from (Perera et al., 2012)).**

A. In healthy control skin, the epidermal keratinocytes become progressively flatter as they differentiate through the layers from the stratum basale (adjacent to the dermis) via the stratum spinosum and stratum granulosum, and terminally differentiation by becoming corneocytes (these cells lack a nucleus) and forming the stratum corneum. B. In psoriatic lesional epidermis, the keratinocytes do not differentiate completely. This is due to the proliferation of the keratinocyte layer (via dysregulation of the keratinocyte proliferation pathways) which results in the premature maturation of the keratinocyte layer.



disease is required in order to effectively treat this debilitating illness. One remarkable feature of psoriasis is that when a patient goes into remission, the histological phenotype of psoriatic skin returns to that observed in healthy skin (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). As the first clinical manifestation of psoriasis is the formation of plaques on the skin it was believed for many years that psoriasis occurred due to a dysfunctional keratinocyte response (Krueger et al., 1984). While keratinocytes are still believed to play a role in the pathogenesis of psoriasis, it has in recent years become clear that leukocytes play an intrinsic role in this disease (Perera et al., 2012).

#### **1.6.1.1. Impact of disease**

#### **1.6.1.2. Effect on everyday life**

Patients with psoriasis have a reduced quality of life compared with unaffected individuals (Krueger et al., 2001). Patients with psoriasis between the ages of 18-34 most often reported that the aspects of their lives psoriasis negatively impacted on was sexual activities, in addition to sleeping (Krueger et al., 2001). In contrast, patients older than 55 found that everyday activities such as walking or using their hands, in addition to sleeping was the aspect of their lives which was most affected (Krueger et al., 2001). In addition to the practical implications of living with psoriasis, the emotional impact of living with psoriasis is significant (Krueger et al., 2001). 81% of all psoriasis patients surveyed felt embarrassed for others to see their psoriatic lesions. In addition, 88% of patients feared that the disease would become worse (Krueger et al., 2001). 75% of psoriasis patients surveyed felt physically unattractive and more than 50% felt depressed. In addition, many patients reported that they were subject to prejudice. For example, more than 50% of all participants in the survey stated that other individuals believed their condition was contagious (Krueger et al., 2001).

#### **1.6.1.3. Increased risk of mortality**

In addition, patients with psoriasis have a greater risk of mortality compared to healthy individuals (Gelfand et al., 2007). It was observed that patients with severe psoriasis had an increased risk of mortality, whereas patients with mild psoriasis did not (Gelfand et al., 2007). It was shown that the increased risk of death in patients with psoriasis with severe disease was no different between men and women, thus ruling out gender specific differences (Gelfand et al., 2007). It was observed that men with severe psoriasis died approximately 3.5 years earlier than their healthy counterparts, and women with severe disease died 4.4 years before their respective healthy control subjects (Gelfand et al.,

2007). The cause of increased risk of death in patients with severe psoriasis was varied, and included factors such as intake of alcohol and smoking, in addition to comorbidities (discussed later) such as cardiovascular disease and certain types of cancer (Gelfand et al., 2006b, Neimann et al., 2006).

#### **1.6.1.1. Clinical subtypes of psoriasis**

##### **1.6.1.1.2. Psoriasis vulgaris/Chronic plaque psoriasis**

Though most associate the term psoriasis with what is more specifically psoriasis vulgaris/chronic plaque psoriasis, there are a number of different clinical subtypes of psoriasis, and each of them will be briefly discussed in this section. As stated previously, psoriasis vulgaris accounts for approximately 90% of all psoriatic patients (Perera et al., 2012). Psoriasis vulgaris is characterized by the presence of red lesions with silvery white scales, which are well demarcated though they can arise anywhere on the body, they tend to be most commonly found on the elbows, knees and scalp (Perera et al., 2012). Often, newly forming lesions are first observed by the presence of papules (a solid elevation of skin) (Naldi and Gambini, 2007). During the development of the lesion, the papules grow in size and eventually develop into psoriatic lesions (Naldi and Gambini, 2007). Individual's lesions tend to be static over time.

##### **1.6.1.1.3. Guttate psoriasis**

Guttate psoriasis often occurs after the affected individual has recently cleared a streptococcal throat infection (Naldi et al., 2001, Naldi and Gambini, 2007). Patients who develop this form of psoriasis often have small red papules forming on the extremities and the trunk. Although usually the disease is self-limiting, Guttate psoriasis can lead to the development of chronic psoriasis. There is evidence suggesting that guttate psoriasis is closely related to psoriasis vulgaris, and a strong association was found between guttate psoriasis and PSORS1 (the major genetic determinant of psoriasis vulgaris)(Sagoo et al., 2004). In addition, patients with active inflammatory lesions in psoriasis vulgaris have also reported flares resembling guttate psoriasis.

##### **1.6.1.1.4.. Pustular psoriasis**

Pustular psoriasis is a form of psoriasis that accounts for 2-5% of cases of psoriasis, and is characterized by the appearance of sterile pustules (an elevation of the skin containing inflammatory cells) that contain neutrophils (Naldi and Gambini, 2007). Pustular psoriasis

can be broadly divided into one of two subtypes, localised pustular psoriasis and generalized pustular psoriasis (Naldi and Gambini, 2007).

#### **1.6.1.1.5. Localized pustular psoriasis**

Within the description of localised pustular psoriasis, there are two main subtypes would within this definition, palmoplantar pustulosis and acrodermatitis continua of Hallopeau (Naldi and Gambini, 2007). Patients with palmoplantar pustulosis have thickening of the stratum corneum (hyperkeratosis) affecting palms and soles, in addition to pustule formation on the ventral side of the feet and hands (Naldi and Gambini, 2007). Unlike other psoriatic subtypes such as guttate psoriasis, patients with palmoplantar pustulosis do not develop psoriasis vulgaris. Recent data has shown that palmoplantar pustulosis is not associated with PSORS1 (Asumalahti et al., 2003), which would support the initial findings demonstrating a lack of direct link between palmoplantar pustulosis and psoriasis vulgaris (Naldi and Gambini, 2007).

Acrodermatitis continua of Hallopeau is a rare form of psoriasis which occurs on the fingers and toes (Naldi and Gambini, 2007) and is best described as an eruption of pustules, which can in some cases, spread to the hands, feet and forearms if the pustules unite to form what is sometimes referred to as ‘lakes of pus’ (Rosenberg and Strober, 2004). The formation of Acrodermatitis continua of Hallopeau can occur after the trauma of a single finger or toe, and can lead to osteolysis of the affected fingers and toes (Rosenberg and Strober, 2004).

#### **1.6.1.1.6. Generalized pustular psoriasis**

Patients who develop generalized pustular psoriasis can also have active psoriatic plaques prior to development. During the onset of acute generalized pustular psoriasis (the von Zumbusch type), the patient’s skin becomes sensitive and erythematous (Rosenberg and Strober, 2004). Large numbers of small pustules (often the size of pinheads) are found on the erythematous skin, and these pustules can become confluent forming ‘lakes of pus’, similar to that observed in Acrodermatitis continua of Hallopeau. The pustules will then dry out leaves erythematous skin, on which the patient can subsequently develop more pustules (Rosenberg and Strober, 2004).

#### **1.6.1.1.7. Erythrodermic psoriasis**

Chronic psoriatic plaques tend to be fairly stable. However, erythrodermic psoriasis is a far more unstable form of psoriasis, therefore can lead to the entire body becoming involved,

with patients often displaying diffuse erythema (Naldi and Gambini, 2007). During the development of erythrodermic psoriasis, the major clinical features of plaque psoriasis (i.e. well demarcated inflammatory skin lesions) are no longer found (Balasubramaniam and Berth-Jones, 2004). This can be a rapidly fatal form of psoriasis, and initial management of the disease is essential.

#### **1.6.1.1.8. Nail abnormalities**

Approximately 20% of psoriasis patients have associated changes in their nails (Naldi and Gambini, 2007). These changes include infection of the skin where the base of the nail meets the finger or toe (paronychia), yellow discolouration of the nail, and detachment of the nail from the nail bed (onycholysis) (Salomon et al., 2003). While many advances in the treatment of skin psoriasis have been made, there is a comparative lack of advancements in the treatment of nail psoriasis (Jiaravuthisan et al., 2007).

#### **1.6.1.2. Measurement of disease severity**

##### **1.6.1.2.1. Psoriasis Activity and Severity Index (PASI) score**

The most commonly used method to calculate the severity of psoriasis, especially in clinical trials, is the PASI score. The PASI score was first used in 1978 after Fredricksson and Pettersson developed the score system as a measure of clinical severity in psoriasis (Fredriksson and Pettersson, 1978). To calculate a patient's PASI score, the head, arms, legs and trunk are examined for the following;

- Percentage of area covered with lesions
- Redness (Erythema) of the skin
- Thickness (Induration) of the skin
- Scaling (Desquamation) of the skin

Once these parameters have been measured, the patient is given a score between 0 (no disease) to 72 (Maximal disease) on the basis of the information above (Fredriksson and Pettersson, 1978). The severity of the disease, as measured by PASI score is often used to determine the course of treatment for the patient. E.g. patients with psoriasis with a PASI score greater than 10 will be given biologicals.

##### **1.6.1.2.2. Dermatology Life Quality Index (DLQI)**

Another system to determine the quality of life a patient has while living with a variety of cutaneous disease, including psoriasis, is the Dermatology Life Quality Index (DLQI).

This was first used in 1994 (Finlay and Khan, 1994). This takes the form of a questionnaire and from the answers received from the patient, the effect psoriasis has on the patient's life can be quantified. DLQI is scored 0-30, with 0 – 5 meaning that the disease has no effect, to a small effect on the patient's life, compared with 21-30, which means the disease has a substantial effect on the patient's life (Finlay and Khan, 1994) (also see the weblink below).

<http://www.dermatology.org.uk/quality/dlqi/quality-dlqi-info.html>

#### **1.6.1.3. Comorbidities**

Patients with psoriasis are more likely compared to healthy individuals to suffer from other diseases, referred to as comorbidities (Gottlieb et al., 2008). A variety of comorbidities have been associated with psoriasis including depression, cardiovascular disease and cancer. These will each be briefly discussed below.

##### **1.6.1.3.1. Depression**

Patients with a variety of inflammatory cutaneous diseases, including psoriasis, are known to suffer from depression (Gottlieb et al., 2008, Krueger et al., 2001). Ellis and colleagues reported that that the more severe the disease, the more likely the patient is to be depressed and/or become suicidal (Gupta et al., 1993). Patients with severe psoriasis, which would come with increased percentage body coverage with psoriatic lesions, would most likely be very insecure about their appearance. According to one report, the reasons, which lead to the patient becoming depressed, include fatigue and the perception that they are helpless (Evers et al., 2005). As there is no cure for psoriasis, and patients can have chronic lesions for long periods of time, it is easy to understand how these feelings could manifest themselves.

##### **1.6.1.3.2. Cardiovascular disease (CVD)**

Several studies have shown an association between patients with psoriasis and a greater chance of developing CVD (Gottlieb et al., 2008). One study examined more than 100,000 psoriasis patients and greater than 500,000 matched non-psoriatic patients to examine if there was any association between psoriasis and myocardial infarction (MI) (Gelfand et al., 2006a). It was found that patients with psoriasis with mild or severe disease had a significantly greater chance of MI compared with non-psoriatic patients. In addition, this study also noted that older psoriasis patients had a reduced chance of developing MI compared with younger psoriasis patients (Gelfand et al., 2006a). In agreement with these findings, Stahle-Backdahl and colleagues reported that patients with psoriasis who had

been hospitalized as a result of the disease had a 50% greater chance of death from CVD compared to the general population (Mallbris et al., 2004). Interestingly, patients from outpatient clinics (i.e. patients who are not admitted to stay overnight) did not have a greater chance of developing CVD (Mallbris et al., 2004). These studies imply that severe inflammation may be a factor in predisposing patients with psoriasis to developing CVD.

#### **1.6.1.3.3. Cancer**

Studies have also shown that patients with psoriasis have a greater chance of developing a variety of cancers (Gottlieb et al., 2008). In one study, 9773 patients were followed up after their initial hospitalization as a result of psoriasis. It was found that the incidence of squamous cell carcinoma of the skin, liver cancer, esophagus cancer and oral cancer was increased in these patients compared to the general population (Boffetta et al., 2001). Another report of a Danish cohort found that patients with psoriasis had a significantly higher chance of developing non-melanoma skin cancer, in addition to connective tissue cancers, and cancer of the pharynx, colon and larynx (Frentz and Olsen, 1999).

One of the factors, which could predispose psoriasis patients to developing cancer, is the therapy used to treat psoriasis. Psoriasis patients receiving low doses of PUVA (8-methoxypsoralen plus ultraviolet A) had significantly less chance of developing basal cell carcinoma and squamous cell carcinoma compared to psoriasis patients receiving high doses of PUVA (Nijsten and Stern, 2003). In addition, a study lasting 15 years also reported that the chance of psoriasis patients on high doses of PUVA developing basal cell carcinoma and squamous cell carcinoma was significantly greater compared with psoriasis patients on low doses of PUVA (Stern et al., 1997). However, some of these findings have recently been called into question. Specifically, a study performed in Sweden reported that there was no increase in the risk of developing melanoma in cohort of psoriasis patients who had been on long term PUVA treatment (Lindelof et al., 1999). However, this study did confirm that long term PUVA was associated with a greater risk of developing squamous cell carcinoma.

#### **1.6.1.3.4. Metabolic syndrome**

Metabolic syndrome is the term used to describe several risk factors that can predispose increase an individual's chances of developing diseases such as cardiovascular disease and type 2 diabetes (Gottlieb et al., 2008). The major factors involved in the development of metabolic syndrome are as listed below (Gottlieb et al., 2008);

- Cholesterol and hyperglyceridemia (high levels of triglycerides)

- Increased blood pressure
- Atherogenic dyslipidemia (reduction in high-density lipoprotein)
- Abdominal obesity
- Resistance to insulin/increased levels of glucose while fasting.

Patients exhibiting three or more of these characteristics are classed as having metabolic syndrome according to the National Cholesterol Education Program Adult Treatment Panel III (Warnick et al., 2002).

Several papers have suggested that patients with psoriasis have a greater propensity to developing metabolic syndrome compared to non-psoriatic patients (Mallbris et al., 2006, Sommer et al., 2006, Neimann et al., 2006). Weichenthal and colleagues reported that patients with severe psoriasis, or who were resistant to various types of treatment, had a greater chance of having metabolic syndrome compared to non-psoriatic patients (Sommer et al., 2006). The elevated risk of developing metabolic syndrome in patients with psoriasis occurred between the ages of 40 – 49, and this risk increased as the patients became older. A separate study showed that patients with psoriasis from Europe also showed that patients with psoriasis with severe disease were more likely to develop metabolic syndrome compared to patients with psoriasis with milder disease (Gottlieb et al., 2008).

### **1.6.2. Mouse models of Psoriasis**

At present there are no mouse models that accurately reflect the pathogenesis of psoriasis in humans. (Wagner et al., 2010). Therefore, a lot of the early work and assumptions about the course of disease in psoriasis came from histological analysis of patient biopsies, in addition to investigating various soluble factors found in psoriasis plasma, and the efficacy of various treatments on disease progression in clinical trials (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). In recent years, many mouse models that have a psoriasis-like phenotype have been developed (Wagner et al., 2010). While none recapitulate all of the features noted in patients, these mouse models can be used to study specific aspects of psoriasis pathogenesis.

#### **1.6.2.1. Spontaneous mouse models**

The first mouse models with a psoriasis-like phenotype that were analysed were spontaneous mouse models, one such spontaneous model is the flaky skin mutation ( $Ttc7^{fsn}/Ttc7^{fsn}$ ) mice (Sundberg et al., 1997). Spontaneous mouse models displayed some of the features of psoriasis such as epidermal thickening and leukocyte infiltration. However, there was a general lack of T-cells in the inflammatory infiltrate (Wagner et al.,

2010). Perhaps not surprisingly anti-psoriasis therapies targeting T-cells had little effect on the psoriasis-like phenotype of these spontaneous mouse models of psoriasis. As T-cells are established as being one of the central immune cells involved in psoriasis pathogenesis, these models are considered to be of only limited use and are therefore not discussed further.

#### **1.6.2.2. Transgenic mouse models**

To examine the role(s) a particular gene has in psoriasis, a number of genes have been over-expressed in mice. The gene being expressed is often over-expressed in the basal keratinocytes under the control of a promoter functioning on these cell types (Wagner et al., 2010). Examples of such promoters used for this purpose are Keratin 14 (K14) and Keratin 5 (K5) (Wagner et al., 2010). Both IL-12 and IL-23 have been found at elevated levels in psoriasis lesions compared with healthy skin (Yawalkar et al., 1998, Lee et al., 2004). Mice over-expressing p40, the common subunit of IL-12 and IL-23 in the basal keratinocytes under the control of the K14 promoter, developed an inflammatory skin pathology that was similar to psoriasis in many respects (Kopp et al., 2001). There was leukocyte infiltration of the lesions consisting of T-cells, macrophages, mast cells, neutrophils and eosinophils (Kopp et al., 2001). In addition, thickening of the stratum corneum (hyperkeratosis) and production of the inflammatory cytokines TNF $\alpha$  and IL-1 $\alpha$  by the epidermal keratinocytes was also noted (Kopp et al., 2001).

A recently published mouse model of psoriasiform pathology is the S100a7/a15 double transgenic mouse model (Wolf et al., 2010). The S100 family of proteins have been found to be elevated in psoriasis, therefore Yuspa and colleagues investigated the functional link between S100 proteins, and psoriasis pathogenesis by over-expressing S100a7 and S100a15 in the epidermis, under the control of the keratin 5 promoter (Wolf et al., 2010). These S100a7/a15 mice were 'primed' for lesion development, requiring only stimulation of the skin with a phorbol ester to induce psoriasiform pathology. This psoriasis like phenotype included the formation of Munro's microabscesses in the epidermis, in addition to a dense inflammatory infiltrate in the dermis (Wolf et al., 2010).

Subcutaneous injections of IL-23 and IL-21 can also result in a psoriasis-like pathology in mice (Zheng et al., 2007, Chan et al., 2006). Recently a new mouse model of psoriasis has been described using the TLR7 agonist Imiquimod (van der Fits et al., 2009). In this model, Imiquimod (also called Aldara) is applied to the shaved back of the mouse daily for 5-6 days, and this is sufficient to induce a psoriasiform pathology (van der Fits et al.,



2009). Interestingly, a recently published article has suggested that the inflamed skin pathology observed using Imiquimod can be achieved using TLR7 KO mice (Walter et al., 2013). This study concluded that a component of the vehicle, isostearic acid, is at least partly responsible for the skin inflammation observed upon application of Imiquimod to the shaved back of mice (Walter et al., 2013).

#### **1.6.2.3. Gene knock-out mouse models**

Use of mouse models deficient for the gene(s) of interest, are preferable to transgenic over-expression mouse models. KO models allow an examination of the effect a deletion of any one gene has on the mouse both during development, and during adulthood, both in homeostasis, and inflammation. By over-expressing a gene at a particular site, you are exaggerating the effect this gene may have. One such KO mouse model of psoriasiform pathology, is the D6 KO mouse model developed by our laboratory (Jamieson et al., 2005). This is discussed in further detail in section 1.9.4.1.

Components of the AP-1 transcription factor have been reported to have a role in psoriasis, due to the development of psoriasis-like disease upon genetic deletion of these proteins (Zenz et al., 2005). JunB expression (component of the AP-1 transcription factor), was found throughout the epidermis of uninvolved psoriatic skin. In lesional skin, JunB expression was markedly reduced compared to uninvolved skin, suggestive of a loss of JunB expression being associated with the development of psoriasis lesions. c-Jun (a potential antagonist of JunB), however was strongly expressed in both uninvolved, and lesional psoriatic skin (Zenz et al., 2005). To probe the role of JunB and c-Jun further, an inducible double KO (deletion of both JunB and c-Jun) mouse was generated (Zenz et al., 2005). Upon inducible deletion of both genes, a psoriasiform pathology was observed including hyperkeratosis, and parakeratosis, in addition to infiltration of T-cells into the epidermis, and the formation of Munro's microabscesses. In addition, 'psoriatic-arthritis like' symptoms (psoriatic arthritis develops in many psoriasis patients) were also observed (Zenz et al., 2005). From a molecular standpoint, a variety of cytokines and chemokines were also elevated in the cutaneous mouse lesions, including IL-1 $\alpha$ , IL-1 $\beta$  IFN $\gamma$ , CXCL2, CCL2 CCL3, CCL4 and CXCL10 (Zenz et al., 2005). These inflammatory mediators have been shown previously to be elevated in psoriasis pathogenesis (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012).

#### **1.6.2.4. Xenograft transplantation models**

A xenotransplantation model has been developed that most accurately reflects the phenotype of psoriatic skin (Boyman et al., 2004). Uninvolved psoriatic skin is grafted onto AGR129 mice, and these mice subsequently develop psoriatic lesions. AGR129 mice are deficient in recombinaase activating gene-2, in addition to the type I and type II IFN receptors. This model conclusively showed that skin resident T-cells, in addition to TNF $\alpha$ , are required for the development of psoriasis as anti-CD3 blocking antibodies ameliorated the disease (Boyman et al., 2004). In addition, skin transplanted from healthy controls onto AGR129 mice did not result in lesion formation (Boyman et al., 2004). These data fit with the hypothesis that it is skin resident T-cells in uninvolved psoriatic skin causing this phenotype (Mak et al., 2009). There are more skin resident T-cells found in uninvolved psoriatic skin compared to healthy control skin. However this is not likely to be the reason for the development of the psoriasis-like pathology as injection of large numbers of autologous T-cells from healthy control patients does not result in psoriasiform pathology (Nestle et al., 2005). Activating T-cells from healthy control patients, and injecting them did induce a mild inflammatory response including keratinocyte proliferation, most likely due to cytokine production from the activated T-cells, stimulating the keratinocytes to proliferate. However when autologous T-cells from psoriasis patients are injected it resulted in a psoriatic like phenotype, most likely due to T-cells specific for potential autoantigens found in the lesional tissue (Nestle et al., 2005). This model was used to define a role for pDCs (see section 1.6.9. for more detail) in the initiation of lesion development (Nestle et al., 2005).

At present xenograft models reflect most accurately the phenotype observed in psoriasis patients (Wagner et al., 2010). However, the major drawbacks of this model are the technical difficulties with setting it up. Specifically, to avoid damage to the tissue, the skin from psoriasis patients must be grafted immediately onto the mice. This procedure also requires multiple biopsies from patients and the ethical implications of asking patients for this must also be considered.

#### **1.6.3. Antimicrobial peptides (AMPs) in psoriasis pathogenesis**

AMPs, along with the epidermis, are the first line of defense in the host immune response against invading pathogens (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). Keratinocytes produce a multitude of AMPs including, LL-37, S100 proteins and beta

defensins (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012, Schroder, 2010). AMPs are able to kill a wide variety of bacterial, fungal and viral pathogens through disruption of the invading pathogen's cell membrane (Lai and Gallo, 2009, Nestle et al., 2009a) While the majority of AMPs are barely detectable in healthy skin, their expression is significantly altered in various cutaneous pathologies including psoriasis and eczema. It had been known for some time that only 7% of psoriasis patients suffered from infections of the skin from bacteria or viruses compared with 30% of eczema sufferers (Christophers and Henseler, 1987). The reason for the disparity in the number of infections between the two diseases was uncertain for many years until AMPs were studied in psoriasis and eczema (Nomura et al., 2003, Ong et al., 2002). Psoriasis lesions were found to stain more intensely for the AMP HBD-2, compared to healthy control or eczema lesions (Ong et al., 2002). In addition, western blot and immunodot blot confirmed initial findings with significantly more HBD-2 and LL-37 protein expression detected in psoriasis lesions, compared with eczema lesions (Ong et al., 2002). Elevated levels of AMPs in psoriasis lesions were also observed through use of QPCR (Ong et al., 2002).

#### **1.6.4. Type 1 Interferons (T1-IFNs)**

The innate immune system has been suggested to have a role in the induction of psoriasis (Sweeney et al., 2011). In particular the antiviral cytokine interferon- $\alpha$  (IFN- $\alpha$ ) has long been known to be present in psoriasis lesions (Schmid et al., 1994). *In situ* hybridization identified weak but detectable IFN- $\alpha$  RNA on psoriatic epidermis that was absent on non-lesional or healthy control skin. IFN- $\beta$  however was undetectable by this method in all of the sections tested (Schmid et al., 1994). In addition, RNA for type I IFN inducible genes MxA and 2-5A synthetase was detected in psoriatic lesional skin, but only weakly, if not at all in non lesional and control skin (Schmid et al., 1994). Horisberger and colleagues then confirmed that MxA was expressed in lesional skin through use of immunohistochemistry (Schmid et al., 1994).

Yao et al., 2008 extended these initial findings by confirming that a variety of IFN- $\alpha$  subtypes (of which there are 20 in total) were significantly elevated at the RNA level in psoriasis lesions compared with healthy controls. In addition, Yao et al., 2008, provided evidence for elevated ISG15 (Type I IFN inducible gene) protein staining in lesional skin compared to healthy controls. In contrast to previous findings (Schmid et al., 1994), Yao et al., 2008 were not only able to detect IFN $\beta$  in psoriatic lesions, they also noted that it was

significantly elevated compared with healthy controls. Altogether, these data provided evidence that IFN- $\alpha$  and IFN- $\beta$  are expressed in psoriatic lesions.

It was observed that cancer patients who were given IFN $\alpha$  injections were prone to developing psoriasis-like lesions (Quesada and Gutterman, 1986, Funk et al., 1991). In all cases it was noted that IFN- $\alpha$  injections would result in either an exacerbation of already existing psoriasis symptoms, or the development of psoriasis at the site of injection, despite the patient having no clinical manifestations of the disease pre-treatment (Funk et al., 1991). Upon termination of IFN- $\alpha$  treatment, the psoriasis-like phenotype in these patients was ameliorated (Funk et al., 1991). What made these observations all the more interesting was that patients with no family history of psoriasis developed psoriasis upon IFN- $\alpha$  treatment (Funk et al., 1991). Therefore, while there are individuals who are genetically pre-disposed to develop psoriasis, the symptoms can, under artificial circumstances, manifest themselves in anyone. While these initial observations were made more than 10 years ago the exact role of IFN- $\alpha$  in psoriasis has until recently been unclear. The major source of IFN- $\alpha$  was subsequently identified as a small population of DCs named plasmacytoid dendritic cells (pDCs) (Siegal et al., 1999, Cella et al., 1999), and these cells and the role IFN- $\alpha$  plays in psoriasis pathogenesis are discussed later. Recently it has been shown that epidermal keratinocytes can also produce type I IFNs in psoriasis lesions (Morizane et al., 2012). This suggests that multiple cell types are involved in producing type I IFNs in psoriasis skin.

#### **1.6.5. Neutrophils in psoriasis**

Neutrophils are polymorphonuclear (multi-lobed) cells that are involved early on during the inflammatory response. The fact that neutrophils are found in psoriasis lesions is perhaps not surprising, as CXCL8 (one of the primary neutrophil chemoattractants) is significantly elevated in psoriasis lesions (Sticherling et al., 1991). Although the role of neutrophils in the pathogenesis of psoriasis has not been fully delineated, neutrophils are found in active lesions, particularly soon after lesion formation (Coimbra et al., 2012). It is believed that factors released by neutrophils upon degranulation (e.g. elastase, cytokines etc), are involved in the pathogenesis of psoriasis (Coimbra et al., 2012). It has been suggested that neutrophils may be involved in the development of the characteristic psoriasis plaques (Meyer-Hoffert et al., 2004). Specifically, it was shown that elastase released by neutrophils induces the proliferation of the epidermal keratinocytes (Meyer-Hoffert et al., 2004). This is of some importance as recent publications have indicated the

proliferation of the epidermis in psoriasis occurs predominantly as a result of cytokine stimulation of the keratinocyte layer (Zheng et al., 2007, Caruso et al., 2009). In addition, increased numbers of neutrophils have been found in both the circulation, and the lesions of psoriasis patients (Coimbra et al., 2012) suggesting that they are involved in some way.

#### **1.6.6. Identification of T-cells as the prominent leukocytes involved in psoriasis pathogenesis**

As discussed previously, it was believed for many years that a dysregulation of keratinocyte differentiation and proliferation was predominantly responsible for the clinical manifestations of psoriasis (Krueger et al., 1984). However, in the late 1980s and early 1990s several clinical studies were published showing that both tacrolimus and cyclosporine, could aid in the treatment of psoriasis (Ellis et al., 1986, Thomson et al., 1991, Baker et al., 1987). Both tacrolimus and cyclosporine are immunosuppressant drugs which function by inhibiting the production of IL-2 by the T-cells, thus inhibiting T-cell proliferation. Specifically, tacrolimus and cyclosporine inhibit calcineurin, which is required for the transcriptional activation of IL-2 (Madan and Griffiths, 2007). In all of these studies, a notably reduced number of T-cells in the lesions were noted (Ellis et al., 1986, Thomson et al., 1991, Baker et al., 1987). Use of cyclosporine in psoriasis patients was found to result in substantial clinical improvement in the majority of patients involved in the trial (Ellis et al., 1986, Baker et al., 1987). Both studies noted that clinical improvement was accompanied by a drop in the numbers of T-cells found in the lesions (Ellis et al., 1986, Baker et al., 1987). These data suggested that it was infiltration of the psoriatic lesions by populations of leukocytes that was causing the psoriatic phenotype, with T-cells presumably playing a central role in disease development.

This work was followed up by use of antibodies targeting CD4 (expressed on helper T-cells), in addition to the use of a protein designed to bind the IL-2 receptor on activated T-cells and block the growth of activated T-cells (Prinz et al., 1991, Gottlieb et al., 1995). As with cyclosporine treatment, there was a significant improvement in the patient's condition in both trials (Prinz et al., 1991, Gottlieb et al., 1995). Moreover Gottlieb et al., 1995 provided new insights into psoriasis pathogenesis. It was observed that reduced acanthosis correlated with lower numbers of T-cells infiltrating the lesions (Gottlieb et al., 1995). A reduction of CD8<sup>+</sup> epidermal infiltrating T-cell numbers closely correlated with amelioration of disease (Gottlieb et al., 1995), and it was suggested that infiltration of the epidermis by CD8<sup>+</sup> T-cells was a major causative factor of the disease. As discussed later, it has subsequently been shown that CD8<sup>+</sup> T-cells infiltrating the epidermis do indeed

have an essential role in the pathogenesis of psoriasis (Conrad et al., 2007) (See section 1.6.7.). Interfering with co-stimulation of autoreactive T-cells with DCs also proved effective in improving the clinical symptoms (Abrams et al., 1999, Abrams et al., 2000).

#### **1.6.7. The role of T-cells in psoriasis**

With the concept of psoriasis as a T-cell mediated disease firmly established (Mak et al., 2009, Nestle et al., 2009b), studies were undertaken in an attempt to characterize the various populations of T-cells found within the psoriatic lesion, and to delineate their roles in disease. Initial data suggested that T-cells found within the psoriatic lesion were predominantly Th1 cells due to their cytokine profile (Uyemura et al., 1993). The RNA profile of lesional psoriatic skin showed strong IFN $\gamma$  production, a signature cytokine of Th1 cells. Elevated production of IL-2 (involved in the proliferation of activated T-cells) was also noted in lesional skin (Uyemura et al., 1993). In contrast, non-lesional psoriatic skin and healthy controls were found to have significantly less IFN $\gamma$  and IL-2 compared with lesional skin (Uyemura et al., 1993). Interestingly it was noted that psoriatic non-lesional skin was found to have elevated TNF $\alpha$  and IL-1 $\beta$  in comparison to healthy controls (Uyemura et al., 1993). These data are suggestive of ongoing low-level inflammation in non-lesional skin.

Further investigations into the T-cell populations in psoriatic skin suggested that the initial Th1 phenotype as characterized by IFN- $\gamma$  production was an oversimplification (Teunissen et al., 1998). Through use of PCR, Bos and colleagues showed that T-cell clones developed from psoriasis lesions produced IL-17 (Teunissen et al., 1998). This observation was complimented by the detection of IL-17 mRNA in whole psoriasis lesional tissue whereas it was found to be absent in non lesional skin (Teunissen et al., 1998). This paper concluded that T-cells infiltrating the psoriatic lesion expressed IL-17 and this could be involved in the amplification of the inflammatory response (Teunissen et al., 1998).

With the initial data that IL-17 was over-expressed in psoriasis lesions (Teunissen et al., 1998), and the identification and characterization of Th17 cells, further studies were undertaken in order to identify roles for Th1 and Th17 cells in psoriasis. Th17 cells are characterized as expressing IL-17 in addition to IL-22 (Hemdan et al., 2010). IL-22 has been known for many years to inhibit the differentiation of human keratinocytes, in addition to inducing acanthosis of reconstituted human epidermis *in vitro* (Hemdan et al., 2010). These data were confirmed and expanded by Zheng et al., 2007, who showed that

IL-22 derived from Th17 cells was directly responsible for the observed acanthosis *in vivo*. It was shown that IL-23 would drive production of IL-22 from Th17 cells. Importantly, IL-23 driven acanthosis was significantly reduced in IL-22 KO mice, thus confirming the essential role for IL-22 in this process *in vivo* (Zheng et al., 2007).

The factor responsible for proliferation of the epidermal keratinocytes, another hallmark of psoriasis has recently been identified. It was shown that IL-21 is involved in the proliferation of the psoriatic epidermis (Caruso et al., 2009). IL-21 is produced by CD4+ T-cells and is known to be involved in inflammation (Caruso et al., 2009, Costanzo et al., 2010), provided data showing that not only did IL-21 stimulation of keratinocytes result in increased proliferation *in vitro*, intradermal injections of IL-21 resulted in epidermal hyperplasia similar to that seen in psoriasis, in addition to dermal and epidermal infiltration of leukocytes. The comparative roles of IL-21 and IL-22 were called into question in this study when it was shown that blocking antibodies to IL-22 did not affect epidermal hyperplasia induced by IL-21 injections (Caruso et al., 2009). However, IL-22 blocking antibodies did inhibit IL-23 driven epidermal hyperplasia as previously reported (Zheng et al., 2007). A recent publication has shown the importance of CD8+T-cell infiltration of the epidermis for psoriasis pathogenesis (Conrad et al., 2007). Using a xenograft mouse model of psoriasis (Boyman et al., 2004), it was observed that T-cells entering the epidermis express the integrin  $\alpha 1\beta 1$  (Conrad et al., 2007). It was shown that blockade of the integrin  $\alpha 1\beta 1$  inhibited the infiltration of the epidermis by T-cells, in addition to inhibiting the development of the psoriasis-like phenotype (Conrad et al., 2007).

#### **1.6.8. Dendritic cells (DCs), the bridge between innate and adaptive immune responses in psoriasis**

Dendritic cells are called antigen presenting cells (APCs) as they take up antigen, process and present it to naïve T-cells allowing an adaptive immune response to occur (Zaba et al., 2009). Although they were first identified in the 1970's (Steinman and Cohn, 1973), it took some time for their fundamental role in the immune response to be fully appreciated (Zaba et al., 2009, Valladeau and Saeland, 2005). Under resting conditions there are several subsets of DCs found in healthy human skin, intraepidermal Langerhans cells, dermal myeloid dendritic cells, in addition to plasmacytoid dendritic cells, although the data are conflicting for this last population (Zaba LC et al 2009). There is another population of DCs that are found in lesional psoriatic skin, referred to as myeloid dermal inflammatory DCs (Zaba et al., 2009). Each of these DC subsets and their involvement in psoriasis pathogenesis are reviewed below.

### **1.6.9. Plasmacytoid Dendritic cells (pDCs)**

pDCs are a unique subset of DCs that are suggested to be involved in the pathogenesis of psoriasis (Nestle et al., 2005). The name plasmacytoid comes from the fact that pDCs morphologically are very similar to antibody secreting plasma cells (Corcoran et al., 2003). pDCs are well characterized in terms of surface markers, CD4+, BDCA-2+, BDCA-4+, CD123+, CD45RA+, CLA+, CD11C- (Albanesi et al., 2010). pDCs express a very specific set of TLRs (TLR7 and TLR9) that are found in the early endosomes, and this is a clue to their primary function (Zaba et al., 2009). Upon endocytosis of viral RNA or unmethylated CpG DNA fragments (binding to TLR7 and TLR9 respectively), pDCs produce large quantities of type I IFNs that aid in the antiviral immune response (Nestle et al., 2009b). It is well established that pDCs are the major type I IFN producing cell as it was shown that pDCs produce up to 10,000 times more type I IFNs than any other cell type (Kadowaki et al., 2000).

There is controversy with regards to whether pDCs are found exclusively in the circulation, or in both the circulation and the skin in healthy individuals. Using IHC, (Nestle et al., 2005) were unable to find any pDCs in the skin of healthy individuals. In uninvolved psoriatic skin however, they were able to detect pDCs infiltrating the skin, and significantly more again within the psoriatic lesion (Nestle et al., 2005). In addition, a significant reduction in circulating pDCs was observed in psoriasis peripheral blood as opposed to healthy controls (Nestle et al., 2005). It was suggested that under resting conditions pDCs remained in the circulation, and under inflammatory conditions pDCs would enter the skin. In contrast to these data, other studies (Ebner et al., 2004, Guttman-Yassky et al., 2007) detected small numbers of pDC in the dermal regions of healthy controls. To complicate matters further, Guttman-Yassky et al., 2007 could not detect any difference in the numbers of pDCs in healthy controls compared with psoriasis lesions. The lack of consistency in these results is all the more confusing as all of the studies concerned use the same marker for pDCs (BDCA-2) in addition to using the same manufacturer, Miltenyi Biotec, for the antibody (Nestle et al., 2005, Ebner et al., 2004, Guttman-Yassky et al., 2007).

Nestle et al., 2005 provided the first direct evidence that pDCs are activated in psoriasis lesions to produce IFN $\alpha$ , which acts on autoreactive T-cells present in the skin, and results in a psoriatic like phenotype. It was observed that pDCs isolated from psoriasis lesions, but not uninvolved psoriatic skin, or peripheral blood leukocytes, expressed high levels of co-stimulatory molecules CD80 and CD86, and maturation marker CD83 (Nestle et al., 2005).



Secondly, type I IFN inducible genes were found to be upregulated in psoriatic lesional skin but not uninvolved psoriatic skin indicating the production of type I IFNs (Nestle et al., 2005). Using the xenograft model described in section 1.6.2.4. (Boyman et al., 2004), it was shown that blocking antibodies to either IFN $\alpha$ / $\beta$  receptor or BDCA-2 reversed the disease (Nestle et al., 2005). Therefore type IFNs, particularly IFN $\alpha$ , are clearly important for the initiation of psoriasis.

Questions still remained as to the specific factor(s) involved in activating the pDCs. As pDCs require viral RNA or pathogenic products, neither of which are usually found in resting human skin, it was until recently unknown how these cells were becoming activated. pDCs are designed specifically to prevent activation against self RNA/DNA as TLR7/9 are intracellular receptors (Barton et al., 2006). Gilliet and colleagues subsequently showed the antimicrobial peptide LL-37, when combined with self-DNA which can be released during skin trauma, could activate pDCs through TLR9 resulting in the production of high levels of IFN $\alpha$  (Lande et al., 2007). Although normally not available to bind to self-DNA due to its location in the endosomal compartments (Barton et al., 2006), the coupling of self DNA to LL-37 resulted in pDCs taking up the LL-37/self DNA complex and retaining it in the early endosomes. This resulted in pDC activation (through TLR9), IFN $\alpha$  production and subsequent activation of autoreactive T-cells (Lande et al., 2007). The removal of DNA through use of DNase I in the pDC cultures was found to inhibit the production of IFN $\alpha$  thus proving that a complex of LL-37 and self DNA was required for pDC activation to occur (Lande et al., 2007). The current model therefore proposes that trauma to the skin results in damaged/dying cells releasing self DNA into the dermal regions concomitant with enhanced production of LL-37 (Nestle FO et al 2009). Self DNA and LL-37 form a complex and are taken up by pDCs that are found in, and around, the epidermal regions where LL-37 is produced by keratinocytes (Nestle FO et al 2009). pDCs are then activated and produce IFN $\alpha$  which subsequently activates autoreactive T-cells thus initiating psoriasis (Perera et al., 2012).

#### **1.6.10. Resident dermal DCs – Sentinels of the skin**

As stated earlier there are a number of DC subsets found within psoriasis lesions. In healthy skin there is a population of cells named resident myeloid DCs (Zaba et al., 2009). These cells are best phenotyped using BDCA-1/CD1c and were observed to self renew in order to maintain a basal (tissue resident) population of (Bogunovic et al., 2006). This study showed that between 3-5% of mouse and human dermal resident DCs were actively

renewing in order to maintain DC numbers (Bogunovic et al., 2006). In addition these BDCA-1 resident DCs are CD11c<sup>+</sup> CD45<sup>+</sup>CD14<sup>-</sup> (Bogunovic et al., 2006, Zaba et al., 2009). CD11c is an integrin that is expressed on the majority of myeloid DCs, CD45 is found on all haematopoietic cells and CD14 is a monocyte marker. Resident myeloid DCs have limited ability to stimulate a T-cell mediated response although if matured with pro-inflammatory stimuli this ability is significantly enhanced (Zaba et al., 2009). Within the CD11c<sup>+</sup> resident myeloid dermal DC population there exists a sub-population expressing BDCA-3 (CD141). It must be stressed that these cells are BDCA-1 negative (CD1c) and therefore represent a distinct sub-population of cells (Zaba et al., 2009). To summarize, resident myeloid DCs (CD11c<sup>+</sup>, BDCA-1<sup>+</sup> or BDCA-3<sup>+</sup>) are present in non-inflamed tissue, acting as sentinels continually testing antigen in the surrounding environment. The DCs described here are one of two populations of DCs found within the dermal compartment of psoriasis patients. The second population of DCs, called inflammatory DCs are described in section 1.6.11.

#### **1.6.11. Inflammatory dermal DCs in psoriatic skin**

Recently another population of dermal DCs has been identified in psoriasis as being inflammatory in nature. These DCs were phenotyped as being CD11c<sup>+</sup> CD1c/BDCA-1<sup>-</sup> (called inflammatory DCs from now on), and are therefore distinct from resident dermal DCs (CD11c<sup>+</sup> BDCA-1<sup>+</sup>). In psoriasis lesions there is a greater than ten fold increase in these inflammatory DCs compared to healthy controls (Zaba et al., 2009). Notably, this increase in inflammatory DCs in lesional skin was followed by a significant decrease in resident DCs (described in section 1.6.10) (Zaba et al., 2009). These data suggested that resident DCs were being replaced during inflammatory conditions by DC precursors from the circulation as suggested in other studies (Bogunovic et al., 2006, Zaba et al., 2009).

Within the inflammatory DC population found in psoriasis lesions there exist DCs capable of producing TNF $\alpha$  and iNOS (Lowes et al., 2005) which have been named Tip-DCs (tumor necrosis factor and inducible nitric oxide synthase producing DCs). Tip-DCs have the ability to induce the activation and differentiation of Th17 cells which have recently been implicated in psoriasis pathogenesis (Lowes et al., 2005).

#### **1.6.12. Langerhans cells**

Langerhans cells (LCs) are a population of DCs that reside predominantly within the epidermal layers of the skin, and are the first leukocytes that a foreign antigen encounters in the skin (Jariwala, 2007, Johnson-Huang et al., 2009, Zaba et al., 2009). They can be phenotyped using CD1a and CD207/Langerin (Jariwala, 2007, Johnson-Huang et al.,

2009), (Zaba et al., 2009). In psoriasis, it has been observed the ability of LCs to migrate is impaired (Cumberbatch et al., 2006). This study suggested this reduced movement of LCs could have a role in pathogenesis (Cumberbatch et al., 2006). It was subsequently observed that LCs have reduced migratory capacity in uninvolved psoriatic skin of early onset psoriasis patients (i.e. when the disease manifests itself before the patient is 40 years old) (Shaw et al., 2010, Shaw et al., 2012). In contrast, the ability of LCs in uninvolved psoriatic skin to migrate was unaffected in late onset psoriasis patients (Shaw et al., 2010, Shaw et al., 2012). These data suggested the migration capacity of LCs could distinguish between early onset, and late onset psoriasis. Interestingly, it was subsequently shown that LCs from early onset psoriasis patients and healthy controls showed no difference in migration capacity (Shaw et al., 2012). This study concluded that it was the local environment in the epidermis that inhibited the migration of LCs in psoriasis patients, not a defect in the migratory ability of the LCs themselves (Shaw et al., 2012).

#### **1.6.13. The role of macrophages in psoriasis**

Macrophages are another population of antigen presenting cells, found in psoriasis lesions (Perera et al., 2012). Initial studies identified macrophages as belonging to one of two categories, Type 1 (classically activated macrophages) or type 2 (alternatively activated macrophages) (Sica and Mantovani, 2012). Currently the majority of data suggest that the predominant subset of macrophages involved in psoriasis lesions are classically activated macrophages (Sica and Mantovani, 2012). This is to be expected when it is considered that Type 1 macrophages produce IL-23, IL-12, IL-6 and TNF $\alpha$ , which are all found at increased concentrations in psoriasis lesions (Homey and Meller, 2008). There is however a small number of publications that identified type 2 macrophages in psoriasis lesions (Djemadji-Oudjil et al., 1996, Wang et al., 2006).

Two studies have provided direct evidence that macrophages are involved in psoriasis-like skin inflammation in mice (Stratis et al., 2006), Wang H et al 2006). Through co-staining, Wang H et al 2006 showed that macrophages are major producers of TNF $\alpha$ , as TNF $\alpha$  production was found to localize strongly with macrophages. Specific depletion of macrophages through use of clodronated liposomes, or removal of TNF $\alpha$  through administration of etanercept resulted in amelioration of the psoriatic like phenotype (Wang H et al 2006). It was noted that depletion of the macrophages resulted in a significant reduction of TNF $\alpha$  levels in the lesional skin, suggesting that macrophages, and not other cells such as T-cells or DCs, are the major producers of TNF $\alpha$  in psoriasis (Wang H et al

2006). This study provided clear evidence that both CCL2, (required for monocyte/macrophage infiltration of the skin), in addition to TNF $\alpha$  (which can activate macrophages) are required in order to induce a psoriasiform pathology. (Wang H et al 2006).

Stratis A et al 2006 also provided evidence suggesting that macrophages, not T-cells, were responsible for driving the pathogenesis of psoriasis. Similar to Wang H et al 2006, Haase and colleagues noted that both anti-TNF $\alpha$  treatment, and depletion of macrophages ameliorated the disease phenotype (Wang H et al 2006, Stratis A et al 2006).

#### **1.6.14. The role of mast cells in psoriasis**

Mast cells have been known to be involved in the pathogenesis of psoriasis for some time, in particular at the onset of disease (Toruniowa and Jablonska, 1988). The Koebner phenomenon was used to identify a role for mast cells in psoriasis pathogenesis (Toruniowa and Jablonska, 1988). The Koebner phenomenon, initially described in 1877, is the appearance of newly forming psoriatic lesions on skin that was histologically normal (i.e. 'uninvolved'), after minor trauma to the skin (Weiss et al., 2002). Using scarification to induce the Koebner phenomenon in psoriatic patients, it was observed that for the first 72 hours post trauma the number of mast cells found in psoriasis and healthy control skin were similar, although significantly higher than the numbers of mast cells found in non-traumatized skin (Toruniowa and Jablonska, 1988). However, from 4-14 days post-trauma, the numbers of mast cells found in traumatized psoriatic skin continued to increase in contrast to healthy control skin in which the numbers of mast cells remained steady throughout (Toruniowa and Jablonska, 1988).

A striking feature of this study was that, on day 10 post-trauma, mast cells accounted for 21% of all lesional infiltrating leukocytes, providing evidence that mast cells are found in the developing lesion (Toruniowa and Jablonska, 1988). It was noted that mast cells were found in relatively high numbers in the upper dermal layers of psoriatic lesions (Jiang et al., 2001). Lesional mast cells were found at significantly higher numbers compared with mast cells found in either non-lesional skin or healthy controls (Jiang et al., 2001). At present no anti-mast cell therapies are available that act specifically on mast cells in the lesion (Li et al., 2008). However, it has been speculated that cyclosporine, a drug commonly used to inhibit proliferating T-cells could have an inhibitory effect on mast cells through suppression of mast cell degranulation (Li et al., 2008).

### **1.6.15. Therapy in psoriasis**

As discussed in section 1.6.6., anti-T-cell therapies strongly indicated that T-cells have a prominent role to play in the pathogenesis of psoriasis. Therapies have now been developed to target cytokines. These new therapies have an advantage over existing T-cell therapies, as there are other cell types in psoriasis lesions which can produce cytokines involved in psoriasis pathogenesis. For example, macrophages can produce TNF $\alpha$  as discussed in section 1.6.13. At present there are five biological agents (also referred to as ‘biologics’) that target specific molecules involved in psoriasis pathogenesis (Perera et al., 2012). These are recommended for patients with moderate to severe psoriasis (Perera et al., 2012). Three of these target TNF $\alpha$ , and are called Infliximab, Adalimumab and Etanercept (Perera et al., 2012).

#### **1.6.15.1. Anti-TNF $\alpha$ biologics in psoriasis therapy**

The first biologic to be approved for psoriasis patients was etanercept in 2004. Etanercept is a human p75 TNF receptor Fc fusion protein (Perera et al., 2012). Three phase III clinical trials have been performed using etanercept, and all three reported an improvement in the condition of psoriasis patients treated with this biologic (Leonardi et al., 2003, Papp et al., 2005, Tying et al., 2006). Two studies examined the effect of etanercept on the histology of the lesion (Zaba et al., 2007, Gottlieb et al., 2005). The inhibition of differentiation of the keratinocyte layer observed in psoriasis lesions was reversed. There was a reduction in the expression of Th17 associated cytokines and chemokines early on during treatment including IL-17, IL-22 and CCL20 (Zaba et al., 2007, Gottlieb et al., 2005). Later during the treatment regimen, Th1 products such as IFN $\gamma$  were then cleared (Zaba et al., 2007, Gottlieb et al., 2005). Finally the numbers of T-cells and DCs infiltrating the dermis were reduced to levels similar to those found in healthy controls after 12 weeks of treatment (Zaba et al., 2007, Gottlieb et al., 2005).

After three successful phase III clinical trials, Infliximab was approved for use as a therapeutic for psoriasis in the USA and Europe in 2006 (Gottlieb et al., 2004, Reich et al., 2005, Menter et al., 2007). Infliximab is a humanized chimeric anti-TNF $\alpha$  monoclonal antibody that can bind to both the transmembrane bound, and soluble version of TNF $\alpha$ . Similar to the effects of etanercept in psoriasis treatment, infliximab substantially reduces the number of lesion-infiltrating leukocytes, which subsequently reduces keratinocyte proliferation, and the de-differentiation of the keratinocyte layer observed in psoriasis lesions is reversed (Goedkoop et al., 2004).

The most recent anti-TNF $\alpha$  treatment adalimumab was approved for use in psoriasis patients in 2007 (Perera et al., 2012). Similar to etanercept and infliximab, three large phase III clinical trials found that adalimumab ameliorated disease severity in psoriasis patients (Menter et al., 2008, Gordon et al., 2006, Saurat et al., 2008). Normal keratinocyte differentiation was restored, in addition to a reduction in the number of leukocytes in the clearing psoriasis lesion upon treatment with adalimumab (Marble et al., 2007). The number of Langerhans cells found in the clearing psoriasis lesion returned to levels normally observed in healthy skin. Similar to etanercept, Th17 cytokines were reduced first, with Th1 cytokines reduced near the end of disease resolution (Gordon et al., 2005).

#### **1.6.15.2. Anti IL-12/23 biologics in psoriasis therapy**

There are currently two biologics developed to bind to the p40 subunit of IL-12 and IL-23, ustekinumab and briakinumab. Therefore these biologics inhibit cytokines essential for the Th1 (IL-12) and Th17 (IL-23) pathways (Perera et al., 2012). Ustekinumab was approved for psoriasis patients on the back of two successful phase III clinical trials (Leonardi et al., 2008, Papp et al., 2008). Data from initial studies showed a reduction in the gene expression of inflammatory cytokines and chemokines found in psoriasis lesions including TNF $\alpha$ , IL-12/23 p40 subunit, IFN $\gamma$ , in addition to CCL2, CXCL8 and CXCL10 (Toichi et al., 2006). While phase III clinical data are available for briakinumab, this biologic has not yet been approved for therapeutic use in patients (Perera et al., 2012). Further investigation into the molecular mechanisms involved in resolving inflammation in psoriasis upon using anti-IL-12/23 targeting antibodies is now required.

#### **1.6.15.3. Anti-T-cell therapies in psoriasis**

Alefacept, which is a CD2 binding fusion protein was approved for use in psoriasis patients in 2003. This biologic targets predominantly memory T-cells, although it can also bind to DCs. In patients treated with alefacept, DC derived products such as IL-23 and iNOS were significantly reduced (Chamian et al., 2005). Therefore Alefacept can inhibit both T-cells, and DCs.

#### **1.6.16. Genetics in psoriasis pathogenesis**

It was observed in the 1960s that relatives of an individual with psoriasis had a greater chance of developing the condition compared with individuals with no family history of the disease. Monozygotic twins were between 35% - 73% likely to both have psoriasis, when at least one of the twins had the disease (Hellgren, 1967) Dizygotic twins (i.e. non-identical twins) however had a concordance of between 12% - 20%, depending on the

various studies. It was clear therefore, that genetics were partially responsible for pre-disposing individuals to be susceptible to the development of psoriasis. However at no time was 100% concordance noted in monozygotic twins, therefore, other factors were likely to be involved in determining whether an individual developed the disease. Since these initial observations, several psoriasis susceptibility loci have been determined (Perera et al., 2012).

There are 10 established psoriasis susceptibility (PSORS) loci identified to date. PSORS1 is considered to be the most important susceptibility locus, and is found on the long arm of chromosome 6, localizing to a region of 220-kilo bases (kb) in the major histocompatibility complex (MHC) (Perera et al., 2012). PSORS1 first came to the attention of scientists studying psoriasis in the 1970s, as an association between allele HLA-Cw\*602 of the MHC class I molecule HLA-C and psoriasis was observed (Russell et al., 1972, Tiilikainen et al., 1980). Of all psoriasis patients, approximately 60% of them carry the HLA-Cw0602\* allele (Mallon et al., 1999). Individuals with this allele are up to 20 times more likely to develop psoriasis. Clearly, this allele is of some importance in psoriasis pathogenesis. HLA-C is found on a variety of cell types, including DCs. As DCs can present processed antigen to CD8+T-cells, which are found in high numbers within the epidermis in psoriasis, it is not surprising to note that HLA-C is involved in the innate and adaptive immune responses (Perera et al., 2012).

Although PSORS1 is a major susceptibility locus in psoriasis, there are others PSORS which have been identified. These sit out-with the MHC loci. From these loci, more than 10 genes have been identified that are associated with psoriasis pathogenesis (Perera et al., 2012). In particular, genes associated with the Th17 pathway such as IL-23R and IL-12B, which encode the IL-12/23 p40 subunit, and the IL-23 receptor respectively (Duffin and Krueger, 2009).

#### **1.6.17. Chemokines in psoriasis**

As chemokines are essential for leukocyte migration from the circulation into the skin, a greater understanding of chemokines, and their receptors, expressed in psoriasis lesions could lead to a better understanding of the mechanisms of disease. In addition, this knowledge could lead to the targeting of chemokines as a method for attenuating the clinical symptoms of psoriasis. There have been numerous studies that have identified a number of inflammatory CC and CXC chemokines, and their receptors as being up-

regulated in psoriasis lesions (Mabuchi et al., 2012).

Almost all of the chemokines found in psoriasis lesions are produced by keratinocytes, which indicates the important role of these cells in recruiting leukocytes to the plaques (Homey and Meller, 2008). Several chemokines produced by the keratinocytes such as CCL2, CCL5, CXCL1 and CXCL8 (Fantuzzi et al., 2008, Giustizieri et al., 2001, Homey and Meller, 2008, Raychaudhuri et al., 1999) can bind to chemokine receptors expressed on multiple leukocyte populations, indicating that they are involved in bringing in a generic 'leukocyte army' into the lesion to assist with what the body believes to be an infectious agent. As neutrophils are well known to chemotax towards CXCL8, a neutralizing antibody (ABX-IL8) was developed in an attempt to inhibit neutrophil migration into psoriasis lesions (Homey and Meller, 2008). However, blocking the effects of CXCL8 did little to improve patient health. This could be another example of the functional redundancy of inflammatory chemokines, and their receptors.

CXCL9, CXCL10 and CXCL11 have been found to be elevated in psoriasis lesions (Goebeler et al., 1998, Gottlieb et al., 1988, Flier et al., 2001). These chemokines have established roles in recruiting T-cells to sites of inflammation (Groom and Luster, 2011a, Groom and Luster, 2011b). In addition, it has been shown that plasmacytoid DCs are also CXCR3 positive (Chen et al., 2010). As pDCs are important for the initiation of lesion formation in psoriasis (Nestle et al., 2005), and psoriasis is generally considered to be a T-cell mediated disorder, CXCR3 would be a favourable target for potential therapeutics.

There are however, certain chemokines that are up-regulated in psoriasis which may be more useful clinical targets due to the "faithfulness" they have to their receptor (Homey and Meller, 2008). One example of such an interaction is the binding of CCL27 to the receptor CCR10 (Homey et al., 2002, Homey and Meller, 2008, Homey et al., 2000b, Morales et al., 1999). CCL27 is exclusively produced by keratinocytes and its receptor CCR10 is found on almost all (>90%) skin homing CD4<sup>+</sup>T-cells in several cutaneous pathologies including psoriasis (Homey et al., 2002). It was observed that in contrast to inflammatory skin from patients with psoriasis, atopic dermatitis and allergic-contact dermatitis, very few CCR10<sup>+</sup> T-cells were observed in healthy controls suggesting that these cells are recruited under inflammatory conditions (Homey et al., 2002).

Importantly it was noted that there was no major difference in the numbers of CCR10<sup>+</sup> T-cells in any of the cutaneous diseases tested which suggested that CCR10 expression was a



prerequisite for T-cell infiltration of the skin during inflammatory conditions (Homey et al., 2002). As CCL27 expression had been identified on the endothelium (Homey et al., 2002, Homey and Meller, 2008, Homey et al., 2000b, Morales et al., 1999), Zlotnik and colleagues investigated the possibility that CCL27 played a part in adhesion of CCR10<sup>+</sup> T-cells to the endothelium, and subsequent migration into the tissue (Homey et al., 2002). It was subsequently shown that CCL27 binds with high affinity to the dermal endothelial cells and can enhance the adhesion of CCR10<sup>+</sup> T-cells to the endothelium (Homey et al., 2002). It was also noted that *in vivo* neutralization of CCL27 inhibits the recruitment of lymphocytes into the dermis by more than 90%, and conversely intradermal injection of CCL27 resulted in significant recruitment of lymphocytes into the sites of injection (Homey et al., 2002). Therefore, in these models of leukocyte migration, CCL27 and CCR10 appear to have prominent roles. In addition, the production of CCL27 from keratinocytes could be enhanced by the inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$ , both of which are prominent in psoriasis lesions, and could therefore exacerbate CCR10<sup>+</sup> T-cell infiltration into psoriatic lesions (Homey et al., 2002). Increased serum levels of CCL27 were observed in psoriasis patients, and CCL27 expression was significantly reduced in psoriasis patients treated with etanercept (Campanati et al., 2007). These data suggest that CCL27 may have some value as a prognostic marker. However, another study reported that CCL27 expression was significantly reduced when comparing uninvolved psoriatic skin, and lesional psoriatic skin (Riis et al., 2011,). Microarray data also reported a reduction in CCL27 expression in psoriasis lesional skin (Riis et al., 2011, Gudjonsson et al., 2010). As the diseases studied in Homey B et al 2002 are all chronic inflammatory diseases, the enhancement of CCL27 production by inflammatory cytokines could have a role in prolonging CCR10<sup>+</sup> T-cell infiltration into inflamed skin.

The binding of CCL20 to CCR6 is an example of an exclusive chemokine/chemokine receptor binding relationship (Homey et al., 2000a, Liao et al., 1999). It has been shown that CCR6 expression is found in a variety of cell types including memory T-cells, B-cells, (Liao et al., 1999) and Th17 cells (Mabuchi et al., 2012). It was subsequently identified that both CCR6 and CCL20 expression were elevated in psoriasis patients (Homey et al., 2000a). Using both QPCR, and *in situ* hybridization, it was shown that CCL20 RNA was expressed at a significantly higher level in psoriasis keratinocytes compared to healthy controls (Homey et al., 2000a).

An increase in CCL20 protein expression was then confirmed by IHC (Homey et al., 2000a). It was clear that CCL20 had a role in T-cell homing distinct from other

chemokines involved in homeostatic trafficking, as CCL20 expression was absent from both psoriatic non lesional skin and healthy controls (Homey et al., 2000a). As attempts to analyse CCR6 *in situ* was not possible due to a lack of good antibodies, co-staining for T-cells and CCL20 was performed in order to gauge where T-cells are positioned relative to CCL20. It was noted that T-cells were found close to the epidermis, high up in the dermis (Homey et al., 2000a). In addition, intraepidermal T-cells were found in very close proximity with CCL20 staining in the epidermis (Homey et al., 2000a). It may seem counterintuitive that CCR6 appears to have a role in T-cell migration into the dermis with data discussed previously on CCR10's prominent role in T-cell migration into the skin. These data cannot at this time be reconciled. However, the simplest explanation is that the data on CCR10 and CCR6 are simply models of T-cell migration. Whether either of these receptors would have such a prominent role in leukocyte migration in humans is another matter entirely.

Finally it was observed that psoriatic PBMCs could respond to lower concentrations of CCL20 compared with healthy controls, suggesting that they are "primed" in some way to respond to CCL20 and inflammatory mediators like TNF $\alpha$ , IL-1 $\beta$ . (Homey et al., 2000a). This point is especially important as all of these cytokines are known to be over-expressed in psoriatic lesions (Nestle et al., 2009b). In addition, it appears that both CCL20 and CCL27, with established roles in bringing in memory T-cells into psoriasis lesions, are both upregulated upon stimulation with specific pro-inflammatory factors (Homey et al., 2000a, Homey et al., 2002). As TNF $\alpha$  blockers have proven partially successful in treating psoriasis, this gives credence to the idea that psoriasis is a disease in which stopping the activation/recruitment of T-cells is paramount in order to control the disease. Not only does there appear to be a role for CCL20 in the migration of memory T-cells into inflammatory skin, there are currently data which suggest CCL20 also has a role in bringing in immature DCs to the skin (Dieu et al., 1998, Dieu-Nosjean et al., 2000). Immature DCs were found to be more responsive to CCL20 than other DC chemoattractants such as CCL3 and CCL5 (Dieu et al., 1998). This study elegantly showed that upon maturation of the skin infiltrating DCs, the responsiveness to chemokines expressed in the skin is reduced and responsiveness to CCL19 and CCL21, found in the skin, increases (Dieu et al., 1998). It was subsequently identified that CCR6 was highly expressed on immature DCs, therefore the DCs migrated towards CCL20 produced by the keratinocytes. Upon maturation of the DC, CCR6 expression was reduced, and CCR7 expression was elevated, leading to migration of matured DCs to the lymph nodes and the priming of naïve T-cells (Dieu et al., 1998). CCL20 induced migration was not restricted to

DCs differentiated from CD34+ progenitor cells as similar effects were noted on Langerhans cells (Dieu-Nosjean et al., 2000).

In addition to CCL27 and CCL20, CCL17 is also believed to have a role in T-cell homing to inflamed skin (Reiss et al., 2001). Interestingly, in this paper Reiss Y et al 2001 provided strong evidence that the roles of CCL27 and CCL17 are overlapping (Reiss et al., 2001). If an anti-CCL27 antibody was administered to a CCR4 deficient mouse, this resulted in a significant reduction in lymphocyte recruitment to the skin (Reiss et al., 2001). However, this study found that blocking either the interaction of CCL27 with CCR10, or the interaction of CCL17 with CCR4, did not significantly reduce the movement of lymphocytes into the skin (Reiss et al., 2001). Therefore it appears that either CCL17 or CCL27 can recruit lymphocytes effectively and that the functions of these chemokines are overlapping. Although psoriasis is associated with the classic inflammatory chemokines (e.g. CCL2, CCL5, CXCL8 etc as discussed above), emerging evidence suggests that homeostatic chemokines may also be involved in the development of psoriasis. CCR7 and its cognate ligands CCL19 and CCL21 have been discussed previously with regards to their role in T-cell and DC homing to the lymph nodes (Section 1.4.). However, a recent paper has shown that CCL19 and CCR7 were expressed within dermal aggregates in the psoriasis lesion, suggestive of a role in setting up lymphoid-like structures in the skin (Mitsui et al., 2012).

## **1.7. Eczema, a biphasic T-cell mediated disease**

### **1.7.1. Introduction**

Eczema is a chronic inflammatory disease that can manifest itself early on in an individual's life, in most cases during infancy (Werfel, 2009, Hanifin, 2009). Eczema is characterized by the presence of skin lesions that are typically intensely pruritic, and often leads to severe discomfort, loss of sleep and a general reduction in the quality of the patient's life. There are numerous factors known to be involved in the initiation of eczema including various allergens, both food types and inhaled, in addition to infection and irritant substances (Werfel, 2009, Hanifin, 2009). The skin lesions of eczema patients are infiltrated by large numbers of leukocytes that are predominantly T-cells, DCs, eosinophils and macrophages (Werfel, 2009, Hanifin, 2009). In addition, it has recently become clear that the interaction of T-cells with keratinocytes also has a role in the outcome of eczema (Werfel, 2009, Hanifin, 2009). While the differences in eczema lesional skin compared to

skin from a healthy individual are obvious, there are also alterations in non lesional skin from eczema patients (Werfel, 2009, Hanifin, 2009). It was noted that in approximately 30% of all patients examined, a loss-of-function mutation was found in the gene filaggrin, involved in skin barrier formation, which predisposed the individuals to eczema (Palmer et al., 2006).

### **1.7.2. Th1 and Th2 cells in eczema**

The main cell type infiltrating eczema lesional skin are CD4<sup>+</sup>T-cells (Avgerinou et al., 2008, Bieber, 2008, Hanifin, 2009, Werfel, 2009). Initial studies provided evidence that eczema was a Th2 mediated disease as application of allergens epicutaneously resulted in the production of IL-4, IL-5 or IL-13 (Sager et al., 1992, van Reijssen et al., 1992). However, it has subsequently been shown that while Th2 cytokines are required for the onset of eczema lesions, in chronic eczema Th1 cytokines dominate the lesion, in particular IFN $\gamma$  (Grewe et al., 1995, Thepen et al., 1996). It was subsequently shown that treatments for eczema targeting IL-4 and its effects were not successful, but a reduction in the levels of IFN $\gamma$  ameliorated the disease (Grewe et al., 1995, Thepen et al., 1996). In addition it was shown that, in contrast to T-cells isolated after induction of eczema through use of allergens, T-cells obtained from lesions forming spontaneously in eczema patients were found to produce IFN $\gamma$  upon stimulation (Werfel et al., 1996). Thus, Th2 cells appear to be the dominant population in eczema skin initially, but Th1 cells are increasingly important as the disease becomes chronic.

### **1.7.3. Th17 and regulatory T-cells in eczema**

In addition to Th1 and Th2 polarised CD4<sup>+</sup>T-cells, Th17 cells and Tregs have been observed in eczema (Werfel, 2009). Various studies showed that IL-17 mRNA in eczema lesional skin, in addition to Th17 cells in the circulation of eczema patients, were increased compared to healthy controls (Toda et al., 2003, Koga et al., 2008). It was noted in these studies that expression of IL-17 was a feature more common to acute, newly formed lesions compared with established chronic lesions (Koga et al., 2008). It was observed in another study that intraperitoneal injection of ovalbumin would not induce production of IL-17, but epicutaneous administration of ovalbumin would (He et al., 2007). Importantly, epicutaneous administration of ovalbumin resulted in an allergic skin inflammation in mice which resembled the human condition eczema (He et al., 2007). This production of IL-17 was effectively inhibited by the injection of antibodies specific for TGF $\beta$  (required for the induction of Th17 cells in mice) (He et al., 2007). These data are of importance, as

individuals with eczema can be sensitized to allergens epicutaneously (Werfel, 2009). Therefore the work in He et al., 2007 represents a putative mechanism for how IL-17 production occurs early on in the development of eczema. While there is some evidence for IL-17 in the early stages of eczema, the role it plays and the exact molecular mechanisms by which it functions are still elusive.

Natural Regulatory T-cells (nT-regs) are involved in preventing the activation of both autoreactive T-cells, and effector T-cells, and are an essential component of the immune system required to maintain self-tolerance (Sakaguchi et al., 2008). These cells are phenotyped as being CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>. Elevated numbers of these nT-regs have been observed in the circulation of eczema patients compared either with healthy controls or patients with asthma (Ou et al., 2004). In contrast, in eczema lesions, there is an absence of nT-regs (Ou et al., 2004, Verhagen et al., 2006). Therefore it has been suggested that a lack of nT-regs in eczema lesions are partially responsible for the prolonged inflammatory response at this site. However this has recently been a matter of some debate as other studies have identified the presence of natural T-regs in eczema lesions (Schnopp et al., 2007). Another study noted that a mutation in FOXP3 (natural T-reg transcription factor) resulted in a phenotype resembling eczema (Schnopp et al., 2007, Torgerson and Ochs, 2007) in humans, thus providing more evidence for the ability of T-regs to prevent the induction of eczema.

#### **1.7.4. Plasmacytoid Dendritic cells in eczema**

As discussed previously pDCs are a subset of DCs that are prominent in the initiation of psoriasis (see section 1.6.9.). However they appear to have a different role in eczema (Werfel, 2009). While they are found at elevated levels in the circulation of eczema patients, they appear to be absent in eczema lesions (Wollenberg et al., 2002, Novak et al., 2004). pDCs in eczema circulation were found to express the high affinity receptor for IgE, namely FcεRI which was found to be closely associated with multiple IgE molecules (Novak et al., 2004). This study noted that the expression of FcεRI correlated positively with the progression of disease (Novak et al., 2004). Importantly it was observed that aggregation of FcεRI on pDCs resulted in both the reduction of the pDC's ability to produce type I IFNs, in addition to elevated production of IL-10 (Novak et al., 2004). Novak et al., 2004 also noted that pDCs in eczema patients had reduced expression of skin homing molecules including cutaneous lymphocyte antigen-1 (LFA-1) and L-selectin. These data suggest that pDCs are predominantly found in the circulation of eczema

patients due to a reduced capacity to enter the skin. This FcεRI induced inhibition of type I IFN production by pDCs has been speculated to be a factor explaining why eczema patients are particularly susceptible to viral infections (Wollenberg et al., 2002).

#### **1.7.5. Myeloid DCs in eczema**

There are two major subsets of myeloid DCs known to be involved in eczema, Langerhans cells (LC) and Inflammatory Dendritic Epidermal Cells (IDECs) (Novak and Bieber, 2005). LCs are thought to be involved in the initiation of the allergic response in eczema, whereas IDECs are thought to be the dominant myeloid DC subset involved in chronic inflammation (Werfel, 2009). It was shown in various studies that engagement of FcεRI on LCs resulted in the production of CCL2, whereas this did not occur upon FcεRI engagement on IDECs (Novak and Bieber, 2005, Novak et al., 2004). This suggested that LCs are required to recruit monocytes and CD4<sup>+</sup>T-cells into eczema skin. These initial *in vitro* findings were confirmed through isolation of LCs from eczema patient lesional skin, as within six hours of activation of FcεRI, CCL2 production was observed (Novak and Bieber, 2005, Novak et al., 2004). This study suggested that these data provided evidence that LC produced CCL2 was necessary for the recruitment of IDECs to the skin to participate in eczema later in the course of disease (Novak and Bieber, 2005, Novak et al., 2004).

It was also noted that while LCs were the major producers of human CCL2, and therefore primarily responsible for recruitment of various cell types into eczema lesional skin, IDECs were significantly more efficient at inducing T-cell proliferation compared with LCs (Novak and Bieber, 2005, Novak et al., 2004). The potential of IDECs to induce proliferation in T-cells was similar to the production of CCL2 from LCs, as it was dependent on FcεRI ligation, as use of blocking antibodies to this receptor ablated this proliferation inducing capacity (Novak and Bieber, 2005, Novak et al., 2004). In addition, this study found that IDECs obtained from eczema skin had a greater ability to induce T-cell proliferation compared with LCs, thus providing evidence that IDECs are the main DC cell type responsible for inducing T-cell proliferation in eczema lesional skin.

Finally it was observed that LCs primed naïve T-cells to produce IL-4 whereas IDECs induced naïve T-cells to produce IFNγ. (Novak and Bieber, 2005, Novak et al., 2004) This suggested that it was the DCs that partially controlled the biphasic nature of eczema. Therefore initial recruitment of various cell types including IDEC precursors was

predominantly due to LC production of CCL2, and T-cells initially brought into eczema lesional skin would be primed to produce IL-4, thus creating a Th2 environment that is associated with initial eczema disease pathology. Upon recruitment of IDECs however, as these cells have a greater capacity to induce T-cell proliferation, and a tendency to induce IFN $\gamma$  producing T-cells, this would result in a movement away from the initial Th2 environment, to a more Th1 dominated inflammatory response.

#### **1.7.6. Eosinophils in eczema**

It is well established that circulating numbers of eosinophils are elevated in patients with eczema compared with healthy controls (Homey et al., 2006). While eosinophils have been reported to express a variety of chemokine receptors including CXCR3-4, CCR1-5 and CCR9, it appears the interaction of CCR3 and CCL11 is essential for the infiltration of eosinophils into eczema lesional skin (Homey et al., 2006). It was noted by Yawalkar et al., 1999 that both protein expression of CCL11 and CCR3, in addition to the RNA levels of CCL11 and CCR3, were elevated in eczema skin compared to healthy control skin. It was observed however, that CD3<sup>+</sup> T-cells did make up a significant proportion of the cells expressing both CCR3 and CCL11 (Yawalkar et al., 1999). It was suggested by this study that a positive feedback loop was occurring, i.e. Th2 cells expressed CCR3, and would migrate towards the epicenter of CCL11 production in the eczema lesion. Once the Th2 cells entered the eczema lesion, they themselves would produce CCL11. This would allow subsequent migration of Th2 cells, and eosinophils, into the eczema lesion (Yawalkar et al., 1999).

The study undertaken by Baggiolini and colleagues (Yawalkar et al., 1999). was complimented by another study that provided evidence of a requirement for CCR3 in the mobilization of eosinophils from the circulation to eczema lesional skin (Homey et al., 2006, Yawalkar et al., 1999, Ma et al., 2002). It was observed that while CCR3 KO mice sensitized by OVA resulted in the induction of an eczema-like pathology, similar to that of WT mice, eosinophils in eczema lesional skin were virtually absent (Ma et al., 2002). Importantly, the effect a lack of CCR3 appeared to be specific to eosinophils as similar levels of IL-4, IL-5, and IFN $\gamma$  were observed in both WT and CCR3 KO mice. In addition numbers of mononuclear cells and mast cells infiltrating WT or CCR3 KO eczema lesions were unaltered. In addition, OVA sensitized CCR3 KO mice had very similar levels of circulating eosinophils compared with WT mice thus ruling out the possibility of a problem in mobilization of eosinophils from the bone marrow (Ma et al., 2002).

### **1.7.7. Chemokines in eczema**

As chemokines are fundamental components of leukocyte trafficking it is no surprise that many chemokines and their respective receptors are found at elevated levels in eczema patients (Lonsdorf et al., 2009). A recent study by Homey and colleagues showed that eczema lesional skin was strongly associated with marked up-regulation of CCL18 (Pivarsci et al., 2004). Another study noted that eczema patients with elevated IgE levels (approx 80% of all eczema patients) had elevated levels of CCL18 compared with eczema patients (Park et al., 2008). CCL18 is produced mainly by dermal DCs found close to skin infiltrating T-cells (Pivarsci et al., 2004). Until recently, the presumed chemokine receptor by which it mediated its effects remained unknown (Lonsdorf et al., 2009). However, in 2011 it was found, that the transmembrane receptor PITPNM3 mediated the effects of CCL18 in a model of breast cancer (Park et al., 2008). These data have yet to be replicated in another disease setting. As CCL18 is involved in eczema pathogenesis, the relationship between CCL18, and PITPNM3 should be investigated in this context.

Further studies have shown other chemokines to have associations with eczema, one such chemokine is CCL1 (Gombert et al., 2005). Homey and colleagues noted that CCL1 levels were significantly elevated compared with CCL1 produced by a variety of other cutaneous inflammatory pathologies. It was noted that endothelial cells, mast cells and Langerhan's cells were the major producers of CCL1 that acts on its sole chemokine receptor CCR8, found on some subsets of DCs and T-cells. Similar to CCL18, CCL1 production could be up-regulated through exposure of patients to allergens, thus suggesting a prominent involvement in the pathogenesis of eczema (Gombert et al., 2005).

In addition to the association of CCL1 and CCL18 with eczema, CCR4 and CCR10 and their respective ligands have been well studied within the context of eczema (Lonsdorf et al., 2009). (Kakinuma et al., 2003) extended the findings of Homey et al., 2002 by looking at the serum levels of CCL27 in eczema patients. It was shown in this study that the levels of CCL27 in serum increased as the severity of disease increased (Kakinuma et al., 2003). In addition, the levels of serum CCL27 in eczema patients were found to correlate with the severity of disease (Kakinuma et al., 2003).

While CCR10 is expressed on the vast majority of skin homing T-cells, and therefore it is not specific to eczema, expression of other chemokine receptors in the CCR10 positive T-cell population in eczema and psoriasis lesions was found to differ (Vestergaard et al., 2003). In particular, 27% of all CCR10 positive cells in eczema patients were found to be



co-positive for CCR4 whereas CCR4 positive cells were completely absent from psoriasis patients (Vestergaard et al., 2003). Therefore CCR4 is clearly importance for the migration of T-cells into eczema skin.

## **1.8. Atypical chemokine receptors**

In addition to the classical chemokine receptors, there exists a discrete population of chemokine receptors that appear unable to mount a typical signaling response following ligand binding. These receptors are referred to as ‘atypical’. Four atypical chemokine receptors have been identified, D6, CCX-CKR, DARC and CXCR7 (Table 3.) (Graham et al., 2012, Rot and von Andrian, 2004). There are a number of differences between ‘typical’ and ‘atypical’ chemokine receptors. Firstly, unlike classical chemokine receptors, atypical chemokine receptors do not appear to couple ligand binding to signal transduction pathways, and are referred to as “silent receptors”. Secondly, the atypical receptors have an alteration in a conserved motif in the second intracellular loop of all classical, or ‘typical’, chemokine receptors (Table 4.) (Graham et al., 2012, Rot and von Andrian, 2004). It is believed the differences found in this motif prevent these atypical receptors from signaling. When signaling chemokine receptors are mutated in this conserved motif in the second intracellular loop they are unable to signal upon ligand binding (Graham et al., 2012, Rot and von Andrian, 2004). Thirdly, the atypical receptors are unable to mediate cell movement via chemotaxis towards their cognate chemokines (Graham et al., 2012, Rot and von Andrian, 2004). Therefore these atypical receptors are considered to be scavenger receptors with various roles in controlling inflammatory and homeostatic chemokine levels *in vivo*.











## **1.9. D6, an atypical receptor that binds inflammatory CC chemokines**

### **1.9.1. Initial identification and early observations**

The atypical chemokine receptor D6 was cloned in 1997 (Nibbs et al., 1997a, Nibbs et al., 1997b, Bonini et al., 1997). Full length cDNA encoding D6 was produced from the RNA of mouse brain (Nibbs et al., 1997a), and a single open reading frame was identified. Full length D6 protein is 378 amino acids in length and was found to be a seven transmembrane spanning protein containing a conserved cysteine motif that is characteristic of heptahelical

Receptor	Ligands
D6	CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL12, CCL13, CCL14, CCL17, CCL22, CCL23, CCL24
CCX-CKR	CCL19, CCL21, CCL25
DARC	CCL2, CCL5, CCL7, CCL11, CCL13, CCL14, CCL17, CXCL5, CXCL6, CXCL8, CXCL11
CXCR7	CXCL11, CXCL12

**Table 1.3. Ligand binding profiles of the atypical chemokine receptors.**

Typical chemokine receptor		<b>DRYLAIV</b>	
D6		<b>DKYLEIV</b>	
CCXCKR		<b>DRYVAVT</b>	
DARC			
CXCR7		<b>DRYLSIT</b>	

**Table 1.4. Alterations in the second intracellular loop of atypical chemokine receptors.**

receptors (Nibbs et al., 1997a). The same group also subsequently cloned the human form of D6 (Nibbs et al., 1997b). Human D6, which is similar to mouse D6, also contained the conserved cysteine motif (Nibbs et al., 1997a). Further analysis of the amino acid sequences of mouse and human D6 revealed that D6 might differ functionally compared to the other chemokine receptors. One notable difference between D6 and other chemokine receptors was that in the second intracellular loop there was a variation in the highly conserved DRYLAIV motif. In D6 this had been altered to DKYLEIV (Nibbs et al., 1997a, Nibbs et al., 1997b). Alterations in this DRYLAIV motif are a common feature amongst atypical chemokine receptors (Graham et al., 2012, Rot and von Andrian, 2004, Ulvmar et al., 2011). This altered motif affected the ability of D6 to couple its ligand binding to classical signaling cascades as D6 was shown not to induce calcium fluxes upon binding to its ligands (Nibbs et al., 1997b).

To examine D6 expression in mice and humans, northern blots were carried-out. The initial data obtained identified the placenta as an area of strong D6 expression, in addition to weaker but detectable expression of D6 in other sites such as the liver, intestine and leukocytes (Nibbs et al., 1997a, Nibbs et al., 1997b, Bonini et al., 1997). Ligand binding studies demonstrated that D6 can bind to many inflammatory CC chemokines including CCL4, CCL3, CCL2, and CCL8 (Nibbs et al., 1997a, Nibbs et al., 1997b, Bonini et al., 1997). It was noted in these studies that D6 bound to different CC chemokines with markedly different binding affinities (Nibbs et al., 1997a, Nibbs et al., 1997b, Bonini et al., 1997). It was observed that CC chemokines, which D6 bound to with high affinity, had a proline residue at position 2 in the mature sequence. Clearly this proline residue is of some importance in terms of high affinity binding (Nibbs et al., 1999). It was observed by Graham and colleagues, that in humans there are two forms of CCL3/MIP-1 $\alpha$  (Nibbs et al., 1999). These were originally designated MIP-1 $\alpha$ -S and MIP-1 $\alpha$ P, on the basis of whether these isoforms of MIP-1 $\alpha$  have a serine residue (MIP-1 $\alpha$ -S), or a proline residue (MIP-1 $\alpha$ -P) at position 2 in the mature sequence. Importantly, it was shown that MIP-1 $\alpha$ P had a significantly higher binding affinity for D6 compared with MIP-1 $\alpha$ -S (Nibbs et al., 1999).

This work was extended by the finding that while a proline residue at position 2 in the mature sequence of D6 ligands is required for D6 mediated degradation, it is not essential for binding of the ligand to D6 (Savino et al., 2009). Further studies identified yet more inflammatory CC chemokines that D6 could bind to (Fra et al., 2003, Bonecchi et al., 2004), and at present, all known inflammatory CC chemokines can be bound and internalized by D6 (Graham, 2009). However, D6's ligand binding profile was found to be

restricted to inflammatory CC chemokines as homeostatic CC chemokines, or CXC, XC and CX3C chemokines are not bound by D6 (Graham, 2009). It has also been observed that D6's ability to bind and internalize ligands can be regulated by the peptidase CD26. This peptidase can clip chemokines that contain a proline residue at position 2 in their sequence, which subsequently reduces the chemokine's binding affinity for D6 (Bonecchi et al., 2004). As this proline residue is required for high affinity binding of D6 to its ligands, removal of this proline residue by peptidases significantly reduces the ability of D6 to bind to, and internalize chemokines (Graham, 2009). It is clear from the above studies that a proline residue at position 2 confers some sort of advantage to chemokines binding to D6. The exact reason for this advantage is as yet not clear. It is possible that inflammatory CC chemokines with this proline residue are structurally more compatible with D6's ligand binding sites.

### **1.9.2. An understanding of the molecular mechanisms of D6 function**

Studies by two groups helped define the function of D6 (Fra et al., 2003, Weber et al., 2004). Both groups agreed that while classical chemokine receptors responded to chemokine ligation by significantly reducing the receptors found on the cell surface, ligation of D6 by inflammatory CC chemokines did not significantly alter the levels of D6 expression on the surface of the cell (Weber et al., 2004, Galliera et al., 2004). Another feature of D6 that differed from classical chemokine receptors was that ability of cells transfected with D6 to internalize CCL3 was not significantly affected by pre-incubating these cells with CCL3 (Weber et al., 2004). On the other hand, CCR5 transfected cells were found to have a reduced capacity to internalize CCL3 compared with untreated cells (Weber et al., 2004). In addition it was shown that D6 expression was predominantly intracellular with approximately 95% found within the cell (Weber et al., 2004). Nibbs and colleagues also showed that the majority of D6 is localised to the recycling endosomes (Weber et al., 2004). Another study suggested that D6 localised to the early endosomes as it closely associated with the early endosomal marker EEA1, but not late endosomal marker LAMP1 (Bonecchi et al., 2008, Galliera et al., 2004, Weber et al., 2004). In addition, D6 was found to closely associate with Rab 11, a marker that identifies recycling endosomes (Bonecchi et al., 2008).

There is controversy regarding the role, or indeed lack of role, of  $\beta$ -arrestin in ligand internalization. Weber et al., 2004 through use of dominant negative plasmids for  $\beta$ -arrestin or dynamin provided evidence that expression of the dominant negative dynamin

plasmid significantly reduced ligand uptake, whereas altering  $\beta$ -arrestin expression levels did not perturb ligand uptake. In contrast, Galliera et al., 2004 suggested that  $\beta$ -arrestin is involved in D6 receptor internalization. It was observed that in  $\beta$ -arrestin null MEFs, D6 expression was found predominantly on the plasma membrane compared with  $\beta$ -arrestin positive MEFs in which D6 was found almost exclusively within the cell. (Galliera et al., 2004).

### **1.9.3. D6 expression in humans**

It was noted in the late 90's that lymphatic vessels (LVs) were found to have binding sites for CCL5, CCL2 and CCL7 (Hub and Rot, 1998). This "chemokine signature" corresponded to some of the ligands D6 can bind to (Nibbs et al., 1997a, Nibbs et al., 1997b), therefore the possibility of D6 expression on LVs was examined (Nibbs et al., 2001). This study utilized both D6 specific monoclonal antibodies raised against D6 transfectants, in addition to *in situ* hybridization to examine D6 protein, and D6 RNA expression, on LVs in human tissue (Nibbs et al., 2001). LV localization of D6 was confirmed through use of antibodies specific for podoplanin, a marker for LVs (Breiteneder-Geleff et al., 1999). This report (Nibbs et al., 2001) was fundamental to the current understanding of D6 as it provided clear evidence that D6 is found at a prime location to control the movement of leukocytes between peripheral sites and lymph nodes.

D6 expression has also been identified on a number of leukocyte populations (McKimmie et al., 2008). Use of FACS, in addition to QPCR, identified D6 expression on T-cells, B-cells, mast cells and macrophages in addition to both myeloid and plasmacytoid dendritic cells (McKimmie et al., 2008). The role of D6 on leukocytes is not clear. It has been proposed that D6 on leukocytes is in a better position to control excess inflammatory CC chemokine production due to the motile nature of leukocytes. Therefore, D6 on leukocytes can more efficiently scavenge inflammatory CC chemokines by moving towards the centre of chemokine production, and subsequently internalizing and degrading the chemokines (Graham and McKimmie, 2006). This reduction of inflammatory CC chemokines would enable the leukocytes to then exit the tissue. Hansell et al., 2011 extended the data on leukocyte D6 expression with the finding that D6 activity (the ability of a cell to internalize and degrade inflammatory CC chemokines) was restricted to B1 B cells in resting WT mice (Hansell et al., 2011).

Nibbs et al., 1997b noted that D6 was highly expressed in the placenta using northern blots. These data were complimented by studies that identified placental D6 immunoreactivity, which localised to the syncytiotrophoblast layer of the placenta (Martinez de la Torre et al., 2007, Madigan et al., 2010) (See section 1.9.4.2. for studies examining the function of D6 on the placenta).

#### **1.9.4. In vivo functions of D6**

##### **1.9.4.1. D6 in inflammatory skin disease**

D6 KO mice were generated and showed no resting phenotype (Jamieson, 2005). However, upon application of the phorbol ester TPA to the dorsal skin of D6 KO mice with there was an inflammatory response, the resolution of which, was delayed compared with TPA treated WT mice (Jamieson, 2005). Importantly, the levels of inflammatory CC chemokines in D6 KO mice stimulated with TPA took longer to clear than in WT mice subjected to the same treatment (Jamieson, 2005). These data suggested a role for D6 in resolving CC chemokine driven inflammation.

On a macroscopic level, inflamed D6 KO mice developed a skin pathology (Jamieson, 2005). To examine this pathology further, a histological analysis of inflamed WT and D6 KO mice was undertaken (Jamieson, 2005). There was increased epidermal proliferation, in addition to the de-differentiation of the keratinocyte layer (Jamieson, 2005), similar to the phenotype observed in psoriasis lesions (Mak et al., 2009, Nestle et al., 2009b). The psoriasis-like pathology observed in inflamed D6 KO mice however, was transient, and would resolve spontaneously (Jamieson, 2005) In contrast, the human condition psoriasis is a chronic inflammatory disease, often requiring treatment to control and/or ameliorate the symptoms (Mak et al., 2009, Nestle et al., 2009b). Collectively, these data suggested that D6 has a role in psoriasis pathogenesis.

A second study using inflamed D6 KO mice provided further evidence for a role for D6 in controlling the cutaneous inflammatory response (Martinez de la Torre et al., 2005). Using CFA to induce local inflammation, it was noted that D6 KO mice developed significantly more inflammatory lesions, in addition to greater leukocyte infiltration of the lesions, compared to WT mice (Martinez de la Torre et al., 2005). Leukocyte infiltration in D6 KO mice was not restricted to the lesions, as on day 3 after CFA injection, lymph nodes of D6 KO mice were found to have significantly higher numbers of leukocytes compared with WT mice (Martinez de la Torre et al., 2005). This study also confirmed that there was no

phenotype in WT mice and D6 KO mice in resting conditions (Martinez de la Torre et al., 2005). These data, suggested that D6 has no apparent function during homeostasis, but that it is involved in the context of inflammation (Martinez de la Torre et al., 2005).

#### **1.9.4.2. D6 in controlling chemokine driven inflammation in the placenta**

As D6 is expressed on the syncytiotrophoblast layer of the placenta (Martinez de la Torre et al., 2007, Madigan et al., 2010), the function of D6 at this site was investigated. Martinez de la Torre et al., 2007 provided evidence that inflamed pregnant D6 KO mice displayed higher levels of inflammation induced miscarriage compared with WT mice (Martinez de la Torre et al., 2007). Pregnant D6 KO mice prior to inflammatory stimulus had no differences in the levels of inflammatory CC chemokines found in the placenta when compared with pregnant WT mice (Martinez de la Torre et al., 2007). However, inflammation of D6 KO mice induced significantly greater numbers of T cells and macrophages to move into the placenta compared with WT mice (Martinez de la Torre et al., 2007). This leukocyte invasion of the placenta was significantly reduced when blocking antibodies for inflammatory CC chemokines were used (Martinez de la Torre et al., 2007). These data suggest that inflammatory CC chemokines can cause inflammation-induced miscarriage. Therefore, D6 would appear to be partially responsible for controlling this CC chemokine driven inflammation. The findings were extended by Madigan et al., 2010. This study confirmed D6 immuno-reactivity on the trophoblasts of the placenta, and extended initial observations through use of placenta cell lines by demonstrating D6 function through their ability to internalize CCL2 (Madigan et al., 2010). This study also showed that CCL2 associated with D6 in placental tissue (Madigan et al., 2010).

#### **1.9.4.3. D6 in cancer development**

As discussed in section 1.5.1., there is evidence that chemokines are involved in inflammation driven cancers. As D6 has been shown previously to be involved in resolving the inflammatory CC chemokine response, it is possible that D6 may also be involved in cancer in certain contexts. (Nibbs et al., 2007, Wu et al., 2008). WT mice and D6 KO mice were treated with the mutagen 7,12-dimethylbenz(*a*)anthracene (DMBA), a potent carcinogen, then subjected to multiple TPA stimulations of the dorsal skin (Nibbs et al., 2007). WT mice were found to be predominantly resistant to the formation of papillomas. However, 75% of D6 KO mice treated with DMBA, then TPA, developed papillomas. This study showed that D6 has a role in preventing inflammatory CC chemokine driven papilloma formation. (Nibbs et al., 2007).

With mouse work suggesting a role for D6 in the suppression of papilloma formation, human squamous cell carcinoma (SCC) samples were obtained, and the expression of D6 examined (Nibbs et al., 2007). As seen in a previous study, D6 expression predominantly localised to LVs with occasional leukocyte-like cells also staining positive for D6 (Nibbs et al., 2007). It was noteworthy however that a greater intensity of D6 staining was observed on LVs in all of the SSCs stained compared to control tissue, suggesting that D6 expression is elevated in conditions of inflammation (Nibbs et al., 2007). D6 was then over-expressed on the basal keratinocyte layer, and was found to reduce tumour burden (most likely by reducing CC chemokine driven inflammation) compared to WT mice (Nibbs et al., 2007). The data from this study provided evidence that D6 has a role in reducing inflammatory CC chemokine driven cancer development (Nibbs et al., 2007).

Nibbs et al., 2007 was complimented by another study that focused on breast cancer (Wu et al., 2008). In this study, Shao and colleagues showed that levels of D6 RNA negatively correlated with the invasive potential of various cancer cell lines, i.e. the greater level of D6 RNA, the less the invasive potential of the cell line (Wu et al., 2008). This study also examined patient samples and identified a negative correlation between metastasis to the lymph nodes, and D6 expression (Wu et al., 2008).

#### **1.9.4.4. D6 in gut inflammation**

While expression of D6 on LVs in the gut was identified a number of years ago (Nibbs et al., 2001), the functional significance of D6 expression at this site compared to others was not investigated. Recent studies have extended our knowledge in this area, although again the data are conflicting (Bordon et al., 2009, Vetrano et al., 2010). Gut tissue was obtained from healthy controls, in addition to patients with colon cancer, ulcerative colitis or Crohn's disease, and co-stained for D6, and the lymphatic marker D240 (Vetrano et al., 2010). It was noted that patients with any of the inflammatory gut diseases stated above had elevated expression of D6 on LVs compared to that observed in healthy controls (Vetrano et al., 2010). These data suggest that LV D6 may be elevated in an attempt to regulate, and/or resolve, the inflammatory response in the gut.

To supplement the human pathology work, Vetrano et al., 2010, used the Dextran Sulphate Sodium (DSS) model of colitis in WT or D6 KO mice. It was shown that D6 KO mice developed more severe disease as measured by disease activity index, weight loss and elevated production of inflammatory chemokines and cytokines (Vetrano et al., 2010). Another important observation from this study was that WT mice with colitis had



significantly higher levels of D6 RNA in the inflamed colon compared with non-inflamed WT mouse colon. This observation is important as it backs up the staining in human sections that also showed elevated D6 expression in gut inflammatory disease (Vetrano et al., 2010).

Arguably the most important observation that (Bordon et al., 2009, Vetrano et al., 2010) makes is the confirmation of the prominent role that D6 expression on CD45 negative cells (most likely the LVs) plays in controlling inflammation. D6 KO mice that are reconstituted with bone marrow from WT mice, and therefore express D6 only on leukocytes, developed a colitis phenotype that mirrored the phenotype of D6 KO mice (Vetrano et al., 2010). Whatever the function(s) of D6 on leukocytes, it is clear that stromal D6 is foremost in terms of controlling aberrant inflammatory responses.

While Vetrano et al., 2010 provided evidence that D6 KO mice with colitis have more severe and damaging phenotype, another study described contrasting findings (Bordon et al., 2009, Vetrano et al., 2010). In this paper, it was reported that D6 KO mice did not develop colitis as frequently as WT mice (Bordon et al., 2009). Examination of the histology of the colon, showed that WT mice consistently had a more severe disease phenotype compared with D6 KO mice (Bordon et al., 2009). Notably, there was no difference in either the numbers of leukocytes infiltrating the colons, or in the levels of CCL2, 3, 4 or 5 in either WT or D6 KO mice (Bordon et al., 2009). However, in D6 KO mice, significantly more IL-17A was produced during the later time points of the colitis model (Bordon et al., 2009). In contrast, WT mice used in the colitis study did not have elevated IL-17 levels above those of untreated WT mice. IL-17A blocking antibodies in D6 KO mice with colitis restored the colitis phenotype to something that closely resembled WT mice with colitis (Bordon et al., 2009). The source of elevated IL-17A was identified as  $\gamma\delta$  T cells that were not increased in number in D6 KO compared to WT mice, but had a greater propensity to produce IL-17A (Bordon et al., 2009). There was one set of data that was similar in both studies (Bordon et al., 2009, Vetrano et al., 2010). Both papers showed that WT mice subjected to DSS induced colitis had elevated D6 expression in the colon, compared to vehicle controls (Bordon et al., 2009, Vetrano et al., 2010).

#### **1.9.4.5. Function of D6 on LVs**

It has recently been shown that D6 on LVs has a direct role in the movement of leukocytes from the peripheral tissues to the lymph nodes (Lee et al., 2011). Inflamed D6 KO mice were found to have elevated CCL2 accumulation on their LVs. This resulted in reduced

migration of leukocytes from the peripheral tissue to the lymph nodes, a phenomenon termed “congested lymphatics” (Lee et al., 2011). This study suggested the role of D6 on LVs is to keep the lymphatic surfaces clear of inflammatory CC chemokines, thus allowing efficient movement of leukocytes from the peripheral tissues to the lymph nodes (Lee et al., 2011). The role of D6 on LVs has recently been expanded with a study examining LV-D6 expression in a variety of contexts (McKimmie et al., 2013). This study showed that D6 expression on LVs *in vitro* can be regulated by inflammatory cytokines, in particular IFN $\gamma$  and IL-6 (McKimmie et al., 2013). In addition, it was noted that D6 is highly expressed in the lymphatic cancer Kaposi Sarcoma, possibly to inhibit inflammatory CC chemokine driven responses from inhibiting tumour development (McKimmie et al., 2013).

#### **1.9.4.6. D6 in liver inflammation**

Although expression of D6 in human liver had been identified more than 10 years ago (Nibbs et al., 1997b), until recently there had been no studies focused on dissecting the function of D6 in the liver. Studies on both human and mouse D6 during liver inflammation have recently been published suggesting that D6 is active in controlling inflammation in this organ (Berres et al., 2009, Lin et al., 2011, Wiederholt et al., 2008). Using CCL<sub>4</sub> to induce acute liver injury, a modest elevation in D6 protein expression was noted (Berres et al., 2009). In addition, H+E staining of liver obtained from D6 KO mice with acute liver injury revealed greater liver damage than that seen in WT mice (Berres et al., 2009). Significantly higher levels of inflammatory CC chemokines, and subsequent tissue infiltration of leukocytes, were observed in D6 KO mice compared to WT mice (Berres et al., 2009). This study is yet another example of the involvement of D6 in controlling the CC chemokine driven inflammatory response. A second study by Wasmuth and colleagues identified a number single nucleotide polymorphisms (SNPs) in D6 that positively correlated with liver inflammation (Wiederholt et al., 2008).

#### **1.9.4.7. D6 in diabetes**

To investigate a possible function for D6 in controlling autoimmune diabetes, (Berres et al., 2009, Lin et al., 2011, Wiederholt et al., 2008) generated NOD mice that over-expressed D6 in the pancreatic islets. While NOD mice in various stages of autoimmune diabetes produced various inflammatory CC chemokines, over-expression of D6 in the pancreatic islets resulted in reduced levels of CCL2 and CCL5, both D6 ligands (Berres et al., 2009, Lin et al., 2011, Wiederholt et al., 2008). The numbers of lymphocytes migrating

into the pancreas were also significantly reduced in mice that over-expressed D6 in the pancreatic islets (Berres et al., 2009, Lin et al., 2011, Wiederholt et al., 2008).

#### **1.9.4.8. D6 in *Mycobacterium tuberculosis* infection**

Further evidence that D6 has a role in controlling the inflammatory response was provided in a study of *Mycobacterium tuberculosis* (Mtb) infection using WT and D6 KO mice, in addition to an examination of tissue obtained from patients with Mtb. (Di Liberto et al., 2008) It was observed that mice infected with Mtb lacking D6 had significantly greater mortality compared with WT animals (Di Liberto et al., 2008). Elevated numbers of T-cells and macrophages were observed in the lungs of D6 KO mice compared with WT mice. Moreover, D6 KO mice infected with Mtb were found to have significantly elevated inflammatory CC chemokine and cytokine levels in the serum compared with WT mice (Di Liberto et al., 2008). Interestingly the bacterial loads were similar in WT and D6 KO mice, suggesting that it was not the mycobacterial infection that killed the mice, but rather the prolonged and exaggerated inflammatory response which resulted in multiple organ failure (Di Liberto et al., 2008). The blocking of inflammatory CC chemokines significantly reduced mortality in D6 KO mice although the bacterial loads were significantly increased therefore showing the requirement for CC chemokines to bring in inflammatory leukocytes to control bacterial load (Di Liberto et al., 2008).

#### **1.6.4.9. D6 in transplantation**

A recent publication has provided evidence for a role for D6 in transplantation (Bradford et al., 2010). It was observed by Bradford et al., 2010 that in cardiac rejection, the levels of D6 increase as the severity of the rejection increases. These elevated levels of D6 were concomitant with elevated levels of CCL2 and CCL5. These data suggested that as the levels of inflammation increased, so did the levels of D6 in an attempt to control elevated levels of inflammatory CC chemokines (Bradford et al., 2010).

#### **1.6.4.10. D6 in myocardial infarction**

A recent study into the role of D6 in myocardial infarction has shown that D6 KO mice are more susceptible to cardiac rupture after infarction (Cochain et al., 2012). In addition, tissue remodeling after infarction is dysregulated in D6 KO mice, compared with WT mice (Cochain et al., 2012b). In recently infarcted human tissue, undamaged viable tissue was D6 negative, however D6 expression was detected in inflammatory areas of the infarcted tissue. D6 positive immuno-staining localised to leukocytes and LVs, in addition to necrotic tissue and vascular endothelial cells (Cochain et al., 2012).

#### **1.9.4.11. Unexpected role of D6 in promoting inflammation**

There have been a number of studies that have suggested the primary function of D6 is to promote inflammation, and not to resolve inflammation. Bordon et al., 2009 has already been discussed within the context of another study in gut inflammation in section 1.9.4.4. Another study that broke with the model that a lack of D6 induces a more severe inflammatory response was Liu et al., 2006. D6 KO mice with EAE exhibited significantly reduced disease compared with WT mice with EAE (Liu et al., 2006). The reduced response noted in D6 KO mice was shown to be due to reduced T-cell priming (Liu et al., 2006). Whitehead et al., 2007 observed increased leukocyte numbers in the lungs of D6 KO mice in a mouse model of allergic inflammation, however there was reduced airway reactivity to methacholine in D6 KO mice. This attenuated airway reactivity was in spite of significantly elevated levels of inflammation observed in the lungs of D6 KO mice (Whitehead et al., 2007). This study concluded that the ability of D6 to control lung inflammation was dependent on the concentration of inflammatory CC chemokines present. At high or low levels, D6 was unable to control the level of chemokines, whereas at moderate levels D6 could reduce lung inflammation (Whitehead et al., 2007). It is possible that a chemokine threshold exists that is required for D6 to begin to control the inflammatory response. Once this threshold is reached, D6 begins to control the levels of inflammatory CC chemokines. Above a certain point however, it is possible the ability of D6 to control/resolve inflammatory responses is diminished as D6 is overwhelmed.

A paper has recently been published that suggests that D6 has a role in the regulation of immune responses through its effect on myeloid cells with immunosuppressive potential (Savino et al., 2012). It was shown that D6 KO mice had elevated numbers of circulating Ly6C<sup>high</sup> monocytes. In addition, there was a significant decrease in Ly6C<sup>high</sup> monocytes found in the bone marrow (BM) of D6 KO mice. This reduction of Ly6C<sup>high</sup> monocytes in the BM suggested increased exit and migration, which was confirmed by the finding of elevated Ly6C<sup>high</sup> monocytes in the spleen (Savino et al., 2012). Use of BM chimeras suggested that the loss of D6 expression on non-hematopoietic tissues, most likely the LVs, was responsible for this increase in circulating Ly6C<sup>high</sup> monocytes, as WT mice reconstituted with D6 KO BM did not show elevated circulating Ly6C<sup>high</sup> monocytes (Savino et al., 2012). Mobilisation of Ly6C<sup>high</sup> monocytes from the BM to the spleen was also noted under inflammatory conditions (Savino et al., 2012). This increase Ly6C<sup>high</sup> monocytes in the spleen was dependent on CCR2 as mice reconstituted with CCR2<sup>-/-</sup> cells did not have an accumulation of Ly6C<sup>high</sup> monocytes in the spleen (Savino et al., 2012). Surprisingly, D6 KO Ly6C<sup>high</sup> monocytes, compared to WT Ly6C<sup>high</sup> monocytes, had

enhanced ability to attenuate the production of IFN $\gamma$  from T-cells. A possible *in vivo* function for these Ly6C<sup>high</sup> monocytes was demonstrated in this study, as these cells were able to delay Graft vs host disease (Savino et al., 2012).

An immunohistochemical analysis was recently published detailing the expression of D6 in tissue from chronic obstructive pulmonary disease (COPD). It was observed that D6 expression predominantly localised to the macrophages found in the pulmonary alveolus. Interestingly, D6 expression on alveolar macrophages was found to positively correlate with inflammatory components, including CD8<sup>+</sup>T-cells and TNF $\alpha$ . This study concluded by suggesting that increased D6 expression might have a role in promoting the inflammatory response. (Bazzan et al., 2012).

#### **1.9.4.12. Chemokine independent function of D6**

Recently a role for D6, independent of chemokine scavenging, has been proposed. In Pashover-Schallinger et al., 2012 it was observed that in a mouse model of peritonitis, the numbers of macrophages increased in the peritonea in the D6 KO mice compared to WT mice (Pashover-Schallinger E et al 2012). In addition to identifying D6 expression on human neutrophils, it was noted that in D6 KO mice, the interaction of macrophages with senescent neutrophils was reduced. This study concluded that D6 may have a role in the clearance of neutrophils by macrophages (Pashover-Schallinger et al., 2012). The exact mechanism by which this occurs, has yet to be determined.

## **1.10. CCX-CKR, an atypical receptor that binds homeostatic CC chemokines**

### **1.10.1. Initial identification, and *in vitro* analysis**

Recently a new chemokine receptor was identified that could bind to CC chemokines, although exactly which CC chemokines was initially a point of dispute, (Gosling et al., 2000, Schweickart et al., 2000). Eventually, it was confirmed that this new receptor, named CCX-CKR could bind to CCL19, CCL21 and CCL25 (Gosling et al., 2000, Schweickart et al., 2000). CCX-CKR was suggested to bind weakly to CXCL13 although this remains controversial (Gosling et al., 2000, Schweickart et al., 2000, Townson and Nibbs, 2002). The inability of CCX-CKR to induce signal transduction upon ligand binding led to its classification as an atypical chemokine receptor. Similar to other atypical receptors, there is an alteration in the second intracellular loop (DRYLAIV in classical chemokine

receptors) to DRYWAVT in cows, DRYWAIT in mice and DRYVAVT in humans (Graham et al., 2012, Rot and von Andrian, 2004). It is likely that this impairs/alters the ability of the receptor to signal (Graham et al., 2012, Rot and von Andrian, 2004). The exact function of CCX-CKR is as yet unknown. HEK 293 cells transfected with CCX-CKR internalized CCL19 without a noted reduction in the cell membrane levels of CCX-CKR (Comerford et al., 2006). This contrasts with CCR7, which upon ligand binding was down-regulated from the cell surface (Comerford et al., 2006). Based on these initial observations it appears that CCX-CKR may have a role in regulating the levels of homeostatic CC chemokines and thereby controlling basal trafficking of leukocytes.

Recently CCX-CKR has been shown to impair the response of CXCR3 to its ligand CXCL10 by heterodimerizing with CXCR3 (Vinet et al., 2013). HEK293 cells transfected to express both CCX-CKR and CXCR3, were found to have a significantly reduced migratory potential towards the CXCR3 ligand CXCL10, compared to HEK293 cells transfected with only CXCR3 (Vinet et al., 2013). This study concluded by suggesting the reduced migratory potential of CCX-CKR/CXCR3 co-transfected cells was due to heterodimerization of the receptors (Vinet et al., 2013). Recently there has been a report suggesting that CCX-CKR may make use of  $\beta$ -arrestin for internalization of CCL19 (Watts et al., 2013). In addition, Watts et al., 2013 reported that CCX-CKR may in fact be able to signal. The exact pathways were not elucidated in this study, however, use of  $G\alpha(i)$  inhibitor pertussis toxin resulted in detectable signaling in CCX-CKR cells (Watts et al., 2013).

#### **1.10.2 In vivo analysis of CCX-CKR expression**

Initial studies were hampered by the fact that no antibodies were available for CCX-CKR. Recently there has been a publication reporting the use of CCX-CKR specific antibodies that should aid in identification of the sites of CCX-CKR expression (Takatsuka et al., 2011). RNA levels of CCX-CKR have been detected in immature DCs and activated T-cells in humans, in addition to the heart, gastrointestinal tract and the lungs. (Comerford et al., 2006, Gosling et al., 2000, Schweickart et al., 2000, Townson and Nibbs, 2002). Our understanding of the function of CCX-CKR was enhanced with an elegant study by Bleul and colleagues who “knocked in” GFP to the CCX-CKR locus in order to visualize where the protein was expressed (Heinzel et al., 2007). This study reports that CCX-CKR protein, as determined by GFP expression was not detectable on leukocytes or the heart, and was restricted to stromal cells in the lymph nodes, the epidermis, cells found in the thymus, and possibly LVs of the intestine (Heinzel et al., 2007). It is interesting that CCX-CKR may be

expressed on LVs as D6 is also expressed on these cells (Graham, 2009). Therefore, it is tempting to speculate that D6 and CCX-CKR could orchestrate leukocyte migration through the LVs by regulating both inflammatory, and homeostatic CC chemokines found in the vicinity of LVs.

#### **1.10.3. *In vivo* analysis of CCX-CKR function during homeostasis**

CCX-CKR KO mice were found to develop normally, with no developmental defects observed. However, Heinzl et al., 2007 observed a significant reduction in the numbers of DCs in the skin draining lymph nodes of CCX-CKR KO mice compared with WT mice. In addition, a non-significant increase in the number of DCs in the epidermis was found in CCX-CKR KO mice. Due to the expression of CCX-CKR in the thymus, it was hypothesized that CCX-CKR may be involved in the development of T-lymphocytes. However, Heinzl et al., 2007 found there to be no difference in T-cell development in WT mice and CCX-CKR KO mice. It was observed by over-expressing CCX-CKR in the thymic epithelial cells, a significant reduction in haematopoietic precursor cells were found in the thymus, compared to WT mice. Collectively, these data suggest that CCK-CKR has a role in maintaining the basal trafficking of DCs to the lymph node, and migration of haematopoietic precursor cells to the thymus, under homeostatic conditions.

#### **1.10.4. *In vivo* analysis of CCX-CKR function in disease**

##### **1.10.4.1. CCX-CKR in cancer**

Recent publications have implicated CCX-CKR in cancer (Feng et al., 2009, Zeng et al., 2011). Feng et al., 2009 et al found that CCX-CKR RNA was detectable in a variety of breast cancer cell lines in addition to breast cancer tissue. Stimulation of these breast cancer cell lines with inflammatory cytokines was found to significantly reduce CCX-CKR expression. This study then showed that CCX-CKR reduced the metastatic potential of cancer cell lines *in vitro*. This study finally showed that CCX-CKR reduced tumour growth, and that a negative correlation existed between CCX-CKR expression, and lymph node metastasis (Feng et al., 2009).

These observations were complimented by another study also looking at the expression of CCX-CKR, in addition to D6 and DARC in breast cancer patients (Zeng et al., 2011). This study observed that while expression of all three atypical receptors was found in invasive and non-invasive breast carcinomas, expression in normal breast tissue was rare (Zeng et al., 2011). Similar to Feng et al., 2009, this study also came to the conclusion that

increased expression of CCX-CKR was associated with reduced lymph node metastasis (Zeng et al., 2011). This study also showed that patients who co-expressed all three of the atypical receptors had the greatest survival prospects, with the numbers of patients surviving reduced when only one atypical receptor was expressed. The patients that were found to be negative for all three atypical receptors had the lowest survival rates (Zeng et al., 2011).

#### **1.10.4.2. CCX-CKR in inflammatory disease**

It has recently emerged that in addition to controlling the levels of CCL19 and CCL21, CCX-CKR may also be involved in suppressing aberrant inflammatory responses (Comerford et al., 2010, Bunting et al., 2013). Comerford et al., 2010 showed that CCX-CKR KO mice had three fold more CCL19 and CCL21 in the peripheral lymph nodes, and five fold higher CCL21 protein levels in the circulation compared to WT mice (Comerford et al., 2010). It was also observed that CCX-CKR KO mice immunized with MOG (35-55) peptide emulsified in complete Freund adjuvant (CFA) developed an exaggerated autoimmune disease with increased numbers of Th17 cells, which occurred at an earlier time-point than in WT mice immunized with MOG (35-55) peptide (Comerford et al., 2010). Interestingly, the symptoms of early disease onset could be reversed upon injection of the CCX-CKR KO mice with CCL21. On this evidence, it was suggested that CCX-CKR may have a role in dampening down Th17 mediated inflammatory responses (Comerford et al., 2010).

The findings by Comerford et al., 2010 were extended by another paper from the same group which suggested that CCX-CKR may be involved in the development of thymocytes (Bunting et al., 2013). It was shown that CCX-CKR KO mice have a greater propensity to develop a pathology similar to Sjogren's syndrome, (a T-cell mediated disorder affecting the lacrimal glands, and sweat glands) (Jonsson et al., 2007, Bunting et al., 2013). Specifically, increased lymphocytes were found in both the liver, and the salivary glands (Bunting et al., 2013). This study subsequently identified that CCX-CKR had a role in the development of thymocytes, as this process in CCX-CKR KO mice was dysregulated (Bunting et al., 2013). CCX-CKR has also been investigated in pulmonary sarcoidosis (Kriegova et al., 2006). A significant increase in CCX-CKR RNA expression was observed in bronchoalveolar cells from sarcoidosis patients in comparison with healthy controls (Kriegova et al., 2006). In addition, CCX-CKR immuno-reactivity was found to localize on the ciliated bronchial cells. In addition, these bronchial cells were found to be able to



internalize CCL19 therefore suggesting a functional capability of CCX-CKR at this site (Kriegova et al., 2006).

## **1.11. DARC**

### **1.11.1. Initial findings**

DARC (Duffy Antigen Receptor for Chemokines) was initially identified in the mid-1970s on red blood cells as the receptor used by *Plasmodium vivax* and *Plasmodium Knowlesi* to enter the cell (Miller et al., 1975, Miller et al., 1976). At the time, DARC was known as the Duffy blood group antigen. It was not till the early 1990s that it was recognized that DARC had chemokine binding capability (Chaudhuri et al., 1994, Horuk et al., 1993). It was subsequently shown that DARC can bind to a variety of inflammatory but not homeostatic CXC and CC chemokines (Gardner L et al 2004). DARC expression has been found on blood vessel endothelial cells and red blood cells (RBCs), in addition to purkinje cells, type II pneumocytes and kidney epithelial cells (Rot, 2005). Similar to other atypical receptors there is an alteration in the DRYLAIV motif (Graham et al., 2012, Rot and von Andrian, 2004, Ulvmar et al., 2011). As with the other atypical receptors, calcium flux is not induced as it is with all other classical chemokine receptors.

### **1.11.2. DARC on endothelial cells**

Blood endothelial cell DARC is thought to either neutralize chemokines or have a role in leukocyte extravasation (Graham et al., 2012). There is evidence to suggest that DARC on endothelial cells has a role in the movement of chemokines across cell monolayers (Middleton et al., 1997, Pruenster et al., 2009). This is in contrast with other atypical chemokine receptors, which internalise and degrade chemokines they bind to (Graham et al., 2012,). It has been shown *in vitro*, that chemokines internalized by DARC transfected cells are moved to the opposite side of the cell. This allows the chemokines to be presented at the luminal cell surface, and subsequently enables transendothelial migration of leukocytes to occur (Lee et al., 2003, Pruenster et al., 2009).

Although there is clear evidence suggesting a role for DARC in transcytosis, there have been opposing reports of its function in conditions of inflammation (Dawson et al., 2000, Lee et al., 2003, Luo et al., 2000). In one set of studies, DARC deficient mice were observed to have impaired neutrophil migration upon various inflammatory stimuli (Lee et al., 2003, Luo et al., 2000). However, Dawson et al., 2000 reported the opposite, i.e.

elevated neutrophil infiltration of DARC deficient mice upon inflammatory stimulus. In addition, DARC KO mice are no more susceptible than WT to bacterial infections (Dawson et al., 2000, Mei et al., 2010, Luo et al., 2000, Luo et al., 2000). This is surprising, as it would be expected that the transport of neutrophil chemoattractants to the surface of the blood vessel endothelial cells might be altered in DARC KO mice. With these almost polar opposite phenotypes observed, clearly further studies are required to explain these data sets. Both sets of experiments should be repeated to confirm their results. If the results are confirmed, then any differences between the models used should be carefully examined to exclude the possibility that a minor experimental difference in fact resulted in a marked difference in phenotype.

### **1.11.3. DARC on RBCs**

There are two main ideas as to the function of DARC on RBCs. The first is that DARC removes chemokines from the circulation and was initially called a “chemokine sink”. It was suggested by Shen et al., 2006, that the function of DARC on RBCs was to clear angiogenic CXC chemokines, to inhibit the formation of tumours. Other studies observed that DARC positive humans have greater circulating levels of CCL2 compared to DARC negative humans (Jilma-Stohlawetz et al., 2001, Shen et al., 2006). In addition, injecting inflammatory chemokines in DARC null mice resulted in the loss of chemokine from the circulation far quicker than observed in WT mice (Fukuma et al., 2003). The above data led to the idea that the function of DARC on RBCs is that of a chemokine reservoir, although the data at this point are not clear. It is possible that it is the circulating chemokine levels that dictate the function of DARC on RBCs and endothelial cells. When chemokine levels are low, endothelial cell DARC will be used to allow optimal presentation of chemokines and appropriate leukocyte transcytosis. When chemokines are found at high levels however, DARC on RBCs may predominate and control the levels of inflammatory CXC and CC chemokines in the circulation (Graham et al., 2012).

### **1.11.4. DARC Genome Wide Association Studies (GWAS)**

There has been a number of Genome Wide Association Studies (GWAS) implicating DARC in the pathogenesis of various diseases. GWAS are used to examine whether any differences in genetic traits in various individuals are associated with any particular genetic condition (e.g. susceptibility to a particular disease). Two recent publications suggest that DARC may be involved in obesity (Voruganti et al., 2012, Comuzzie et al., 2012). An association was noted between DARC and the levels of MCP-1 in the circulation. The link between DARC and MCP-1 levels with obesity was made in this study, as obese

individuals often have low-level inflammation (Voruganti et al., 2012). The data in Voruganti et al., 2012 confirms an earlier publication, which implicated DARC in the regulation of MCP-1 levels (Schnabel et al., 2010). Another recent study has provided evidence that DARC may be involved in the regulating levels of lean body mass, which has been used previously to measure skeletal muscle (Hansen et al., 1999) and age (Hai et al., 2012). Another GWAS study has recently shown that while individuals lacking DARC on their RBCs are resistant to *plasmodium vivax* infection, these DARC null individuals had a three fold greater risk of HIV infection compared with healthy controls (Ramsuran et al., 2011).

## **1.12. CXCR7**

### **1.12.1. Initial findings**

Until recently the only known receptor for CXCL12 was CXCR4. However it was believed that another receptor for CXCL12 might exist, and the de-orphanization of RDC1 proved this was the second receptor (Balabanian et al., 2005, Burns et al., 2006, Lee et al., 2003b). Further study showed that CXCR7 can also bind to CXCL11 and it was initially classified as a classical chemokine receptor due to its apparent ability to induce chemokine mediated migration of leukocytes (Balabanian et al., 2005). Schall and colleagues have provided evidence to suggest that in certain contexts, CXCR7 can regulate the migration of CXCR7 positive cells (Zabel et al., 2011). CXCR7 is classified as an atypical chemokine receptor, and like D6, CCX-CKR, and DARC, has an altered DRYLAIV motif in the second intracellular loop. In the case of CXCR7, DRYLAIV is altered to DRYLSIT.

### **1.12.2. Sites of CXCR7 expression**

It appeared that CXCR7 expression is restricted to development and tumourigenesis (Balabanian et al., 2005, Sierro et al., 2007) although initial data suggested expression on lymphocytes (Balabanian et al., 2005). CXCR7 expression on lymphocytes has been a controversial issue. Some groups have contested that CXCR7 is not found on leukocytes (Berahovich et al., 2010b, Cruz-Orengo et al., 2011). However, recent evidence suggests that splenic B-cells express CXCR7 (Humpert et al., 2012). Use of multiple commercially available staining antibodies for CXCR7, in addition to detection of CXCR7 using PCR, mass spectrometry and an uptake assay, all resulted in detection of CXCR7 in splenic B-cells (Humpert et al., 2012). From the studies discussed above, the expression of CXCR7 on leukocytes remains controversial, and further analysis is required.

Through use of a GFP-CXCR7 'knock-in' mouse, it was shown that CXCR7 expression localised to the meninges and microvasculature in brains of both WT mice, and mice with EAE (Cruz-Orengo et al., 2011). This study suggested that leukocytes do not express CXCR7, confirming an earlier report (Berahovich et al., 2010). It was found that CXCR7 expression on endothelial barriers was elevated in EAE compared to control mice, and that this elevation of CXCR7 expression was due to stimulation with T-cell cytokines IL-17 and IL-1 $\beta$  (Cruz-Orengo et al., 2011). This study also showed that CXCR7 is necessary for entry of the leukocytes into the CNS, and that specific antagonism of CXCR7, could block leukocyte infiltration of the CNS (Cruz-Orengo et al., 2011).

A possible reason for the discrepancies in the literature regarding the sites of CXCR7 expression may relate to the various antibodies used to stain for CXCR7 (Berahovich et al., 2010a). At present, there are at least four monoclonal antibodies and seven polyclonal antibodies commercially available for CXCR7 staining. Schall and colleagues, who did much of the early work in describing and analyzing CXCR7, examined the specificity of several of these monoclonal and polyclonal antibodies (Berahovich et al., 2010a). Interestingly, Schall and colleagues found that many of the commercially available antibodies for CXCR7 would either not bind to cells over-expressing CXCR7, or would stain positively for CXCR7 expression in CXCR7 negative sections (Berahovich et al., 2010a). These data highlight the importance of carefully interpreting antibody staining and also the necessity of confirming any positive staining with other methods of detection (e.g. RNA, functional uptake assay etc).

### **1.12.3. Role of CXCR7 in development**

As CXCR7 expression was prominent during development, attempts were made to understand the role(s) CXCR7 plays in this process. It quickly became clear that CXCR7 was essential for normal development as CXCR7 KO mice died shortly after birth with heart defects (Sierro et al., 2007). It was observed that almost all of the CXCR7 null mice (>90%) had defects in the pulmonary valve (Sierro et al., 2007). Another study has also provided evidence for the involvement of CXCR7 in vascular development (Miao et al., 2007). CXCR7 also has a role in the migration of primordial germ cells, in addition to orientating cells moving towards the lateral line primordium (Boidajipour et al., 2008, Valentin et al., 2007). While CXCR4 is also involved in the migration of germ cells, there are functional differences between CXCR4 and CXCR7. CXCR4 is considered to be a signaling chemokine receptor and it therefore controls the movement of germ cells through chemotaxis. CXCR7 compliments CXCR4 by binding and degrading chemokines at the

back of the chemotaxing cell, thus maintaining cell migration in a directional manner (Graham et al., 2012,).

#### **1.12.4. Role of CXCR7 in cancer**

Arguably the most surprising aspect of CXCR7 is that, while its expression is largely restricted to developmental sites (with the exception of endothelial cells, and B-cells as discussed previously), it is frequently associated with tumours, both in tumour cell lines, and primary human and mouse tumours (Calatuzzolo et al., 2011, Miao et al., 2007, Wang et al., 2008). Breast cancer in particular has a close association with CXCR7 as almost all the breast cancer specimens tested were CXCR7 positive (Miao et al., 2007). It was observed that expression of CXCR7 conferred survival and growth advantages compared to CXCR7 negative cells (Burns et al., 2006). Another study showed that CXCR7 enhanced the metastatic potential in lung cancer models (Miao et al., 2007). It was shown by Wang et al., 2008 that increased expression of CXCR7 correlated with increased tumour progression. It has been shown that CXCR7 over-expression in cancer cell lines reduces apoptosis, and increases tumour growth (Wang et al., 2008). Taken together, it is clear that CXCR7 has a prominent role in maintaining, and indeed providing the tumour with a competitive advantage over CXCR7 negative tumour cells. Therefore CXCR7 represents a potential therapeutic target in the context of cancer.

## **Chapter 2 – Materials and Methods**

## **2. Materials and Methods**

### **2.1. Materials**

#### **2.1.1. Antibodies**

##### **2.1.1.1. Primary antibodies**

DAKO (Ely, UK)	Mouse anti-human CD68 monoclonal antibody
DAKO (Ely, UK)	Mouse anti-human CD45 monoclonal antibody
DAKO (Ely, UK)	Mouse anti-human Tryptase monoclonal antibody
R&D systems (Abingdon, UK)	Rat anti-human D6 monoclonal antibody
R&D systems (Abingdon, UK)	Mouse anti-human CCL2 monoclonal antibody
R&D systems (Abingdon, UK)	Goat anti-human CCL5 polyclonal antibody
R&D systems (Abingdon, UK)	Goat anti-human DLEC/BDCA-2 polyclonal antibody
Sigma (Poole, UK)	Rabbit anti-human D6 polyclonal antibody
Sigma (Poole, UK)	Rabbit anti-human Podoplanin polyclonal antibody
Vector Labs (Peterborough, UK)	Mouse anti-human CD3 monoclonal antibody

##### **2.1.1.2. Secondary antibodies**

All secondary antibodies used were obtained from Vector Laboratories (Peterborough, UK).

Biotinylated anti-rat IgG secondary antibody, raised in rabbit

Biotinylated anti-mouse IgG secondary antibody, raised in horse

Biotinylated anti-goat IgG secondary antibody, raised in rabbit

Biotinylated anti-rabbit IgG secondary antibody, raised in goat

##### **2.1.1.3. Isotype controls and IgG fraction controls**

DAKO (Ely, UK)	Rabbit IgG fraction control
DAKO (Ely, UK)	Mouse IgG2b isotype control
DAKO (Ely, UK)	Mouse IgG2a isotype control
DAKO (Ely, UK)	Mouse IgG1 isotype control
R&D systems (Abingdon, UK)	Rat IgG2a isotype control
R&D systems (Abingdon, UK)	Goat IgG control

#### **2.1.1.4. Fluorochromes**

All fluorochromes used were obtained from Vector Laboratories (Peterborough, UK)

Fluorescein isothiocyanate (FITC) Avidin D

Texas Red Avidin D

Cy5 Avidin D

#### **2.1.2. Cytokines and growth factors**

All cytokines and growth factors used were obtained from Peprotech (London, UK)

Recombinant human IL-1 $\alpha$ , IL-6, TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$ , IL-17, IL-20, IL-22, IFN $\alpha$ , IFN $\beta$ .

#### **2.1.3. Serums**

Sigma (Poole, UK)

Normal human serum

Vector Laboratories (Peterborough, UK)

Normal horse serum

Vector Laboratories (Peterborough, UK)

Normal rabbit serum

Vector Laboratories (Peterborough, UK)

Normal goat serum

#### **2.1.4. Tissue culture media**

Gibco (Invitrogen, Paisley, UK)

L-Glutamine

Gibco (Invitrogen, Paisley, UK)

Streptomycin/Penicillin

Gibco (Invitrogen, Paisley, UK)

Foetal Calf Serum (FCS)

PromoCell (Heidelberg, Germany)

Endothelial Cell Growth Medium MV2

PromoCell (Heidelberg, Germany)

Keratinocyte Growth Medium 2 Kit

PromoCell (Heidelberg, Germany)

Trypsin (0.04%) /EDTA (0.03%)

PromoCell (Heidelberg, Germany)

Trypsin inhibitor (0.05%) (0.1% BSA)

PromoCell (Heidelberg, Germany)

Cryo SFM

Sigma (Poole, UK)

RPMI

Starlabs (Milton Keynes, UK)

Molecular biology grade tips

#### **2.1.5. Kits**

Invitrogen (Paisley, UK)

Cytokine human 30plex panel (30plex)

Primer Design (Invitrogen, Paisley, UK)

Nanoscript RT kit

Qiagen (Crawley, UK)

RNeasy mini kit

Qiagen (Crawley, UK)

RNeasy micro kit

Qiagen (Crawley, UK)

RNase free DNase set



Qiagen (Crawley, UK)	Gel extraction kit
Quanta Biosciences VWR (Leicestershire, UK)	Perfecta SYBR green fast mix ROX
Roalab (VH Bio Ltd, Newcastle, UK)	Red PCR master mix tubes
Vector Laboratories (Peterborough, UK)	Avidin/biotin blocking kit
Vector Laboratories (Peterborough, UK)	Hardset Vectashield with DAPI
Vector Laboratories (Peterborough, UK)	ABC kit
Vector Laboratories (Peterborough, UK)	ImmPACT DAB

#### **2.1.6. Chemicals and reagents**

Ambion (Invitrogen, Paisley, UK)	Nuclease free water
Ambion (Invitrogen, Paisley, UK)	RNase Zap
BDH (VWR Leicestershire, UK)	DPX mounting solution
Bioline (London, UK)	5x DNA loading buffer
Bioline (London, UK)	Hyperladder IV
DAKO (Ely, UK)	REAL antibody diluent
Fisher Scientific (Loughborough, UK)	Chloroform
Fisher Scientific	Citric acid
Fisher Scientific	Diaminoethanetetra- acetic acid (EDTA) disodium salt
GE Healthcare (Buckinghamshire, UK)	Ficoll paque
Invitrogen (Paisley, UK)	Ethidium bromide
Invitrogen (Paisley, UK)	Trizol
Sigma (Poole, UK)	Phosphate Buffered Saline (PBS)
Sigma (Poole, UK)	Hydrogen Peroxide (H <sub>2</sub> O <sub>2</sub> )
Sigma (Poole, UK)	2-Mercaptoethanol
Sigma (Poole, UK)	Gentamicin
Sigma (Poole, UK)	Tween 20
VWR (Leicestershire, UK)	Superfrost slides
VWR (Leicestershire, UK)	Ethanol
VWR (Leicestershire, UK)	Xylene

### 2.1.7. Primers

Gene of interest		Primer sequences
Human D6 (inner primers)	Forward	5' AGGAAGGATGCAGTGGTGTG 3'
	Reverse	5' CGGAGCAAGACCATGAGAAG 3'
Human TATA binding protein (TBP)	Forward	5' AGGATAAGAGAGCCACGAACC 3'
	Reverse	5' GCTGGAAAACCCAACTTCTG 3'
Human GAPDH (inner primers)	Forward	5' CAAGGCTGAGAACGGGAAG 3'
	Reverse	5' GGTGGTGAAGACGCCAGT 3'
Human $\beta$ -actin (inner primers)	Forward	5' TAAAACTGGAACGGTGAAGG 3'
	Reverse	5' ATTGTGAACTTTGGGGGATG 3'
Human CCR1 (inner primers)	Forward	5' TTTCTCTTTTGGACCCCCTAC 3'
	Reverse	5' TGTCTGCTCTGCTCACACTCA 3'
Human CCR1 (outer primers)	Forward	5' TGCTAAGACGACCAAATGAGAA 3'
	Reverse	5' ATGCTGTGCCAAGAGTCAGAA 3'
Human CCR2 (inner primers)	Forward	5' AACGAGAGCGGTGAAGAAGT 3'
	Reverse	5' ATGCTGTGCCAAGAGTCAGAA 3'
Human CCR2 (outer primers)	Forward	5' TGAACAAGGACGCATTTCC 3'
	Reverse	5' ATTCCCAAAGACCCACTCATT 3'
Human CCR3 (inner primers)	Forward	5' CAATGTGGTGGTGGTGATG 3'
	Reverse	5' CAGAATGGAAGGGTGACGAG 3'
Human CCR3 (outer primers)	Forward	5' TCACTGCTGAGTTGTATTGGAGA 3'
	Reverse	5' GCGTTTTGATGATTCCTGTGT 3'
Human CCR4 (inner primers)	Forward	5' CACCAAAGAAGGCATCAAGG 3'
	Reverse	5' AACAGGACCAGAACCACCAC 3'
Human CCR4 (outer primers)	Forward	5' ACCCTCGATGAAAGCATATACAG 3'
	Reverse	5' GTTGAGCAGGTACACATCAGTCA 3'
Human CCR5 (inner primers)	Forward	5' TGGTGTAAGAGGATGGGTCTG 3'
	Reverse	5' GCTGGGATGGGAAGGAAA 3'
Human CCR5 (outer primers)	Forward	5' CATCAAGCACAGAAGGAGGAG 3'
	Reverse	5' TGAAATACGGAGGCTGGTAAA 3'
Human CCR6 (inner primers)	Forward	5' TCATCTGCCTTGTTGTGTGG 3'
	Reverse	5' GCCTTGGGTGTTGTATTTTTG 3'
Human CCR6 (outer primers)	Forward	5' GTGCGTGGGTTTTCAGCA 3'
	Reverse	5' GGATGGCTTTGTGCCTTTT 3'

Human CXCR1 (inner primers)	Forward	5' ATCTGTCCCTGCCCTTCTTC 3'
	Reverse	5' CATCCGCCATTTTGCTGT 3'
Human CXCR1 (outer primers)	Forward	5' CAAGGTGAATGGCTGGATTT 3'
	Reverse	5' AAGCAGGAAGATGAGGACGA 3'
Human CXCR2 (inner primers)	Forward	5' ATGGGCAACAATACAGCAAAC 3'
	Reverse	5' GGTGAATCCGTAGCAGAACAG 3'
Human CXCR2 (outer primers)	Forward	5' ACGCACACTGACCCAGAAG 3'
	Reverse	5' AGATGAGGACGACAGCAAAGA 3'
Human CXCR3 (inner primers)	Forward	5' GCCGAGAAAGCAGGGTAGA 3'
	Reverse	5' ATAGAGCAGCGGGTTGAGG 3'
Human CXCR3 (outer primers)	Forward	5' TGCCAATACAAC TTCCCACAG 3'
	Reverse	5' GCAAGAGCAGCATCCACA 3'
Mouse D6 (inner primers)	Forward	5' TTCTCCCCTGCTGCTTCAC 3'
	Reverse	5' TGCCATCTCAACATCACAGA 3'

## **2.2. Methods**

### **2.2.1. Collection of human skin biopsies**

Full thickness biopsies from psoriasis and eczema patients, and healthy individuals were obtained with all ethical approval in place. Upon excision of the biopsy from the patient, the biopsy was cut into two, with half frozen in liquid nitrogen, and half put in formalin. RNA was extracted from the frozen samples using techniques described in section 2.2.3. and 2.2.5. The formalin stored sections were embedded using the Thermo Shandon Histocentre 3. Briefly, samples were incubated in 10% formalin for 30 minutes, then incubated in 70% ethanol, 90% ethanol and 95% ethanol for one hour each, then 5.5 hours in absolute ethanol (100%), 3.5 hours in xylene, and finally incubated for 9 hours in wax. After the tissue fixation, the biopsies were embedded in wax. The 5µm sections were then cut using a microtome, and the sections cut onto superfrost slides.

### **2.2.2. Collection of human PBMC samples**

As with biopsy collection, human peripheral blood samples were obtained with all required ethical approval in place. The PBMCs were then isolated using ficoll mediated density centrifugation as detailed in section 2.2.18.3.

### **2.2.3. RNA extraction from biopsies using trizol**

Biopsies from psoriasis patients, or healthy controls were 'snap-frozen' as described in section 2.2.1., then crushed using a pestle and mortar. The sides of the mortar were scraped with a spatula to ensure any residual sample was not left behind. Once the majority of the liquid nitrogen had evaporated, the mortar was tilted and the sample, and remaining liquid nitrogen transferred to a 15ml falcon tube. Once all the liquid nitrogen had evaporated, 1ml of trizol was added to the homogenate. The tube was flicked, and left at room temperature for 5 minutes. After 5 minutes, if further samples were being processed, the sample was placed on dry ice. The samples were then stored in the -80°C freezer. The next day, samples were thawed on ice, and then transferred into RNase-free Eppendorf tubes. 200µl Chloroform was added to the sample, and the tube was then vigorously shaken, then left at room temperature for 2-3 minutes. The aqueous phase was transferred to a new RNase-free Eppendorf tube, and 500µl of isopropanol was added, and the tubes inverted to mix the samples. The samples were left to incubate at room temperature for 10 minutes. The samples were then spun at 12,000 x g for 10 minutes in a chilled centrifuge at 4°C. The supernatant was removed and discarded, taking care to ensure the pellet was not disturbed. The pellet was then washed using 75% ethanol, by vortexing, and spinning the tube at

7500 x g for 4 minutes at 4°C. The ethanol was then removed, and the pellet allowed to air dry to ensure that any residual ethanol evaporated. Finally, the pellet was dissolved in 100µl of RNase-free water. The extracted RNA sample was then purified and concentrated using an RNeasy mini kit as described in section 2.2.5. A mini kit was used for RNA extracted from biopsies as they are designed to bind to a high concentration of RNA. Micro kits however, are designed for samples where a lower initial RNA yield is obtained. A micro kit was used to purify RNA isolated from primary cells.

#### **2.2.4. RNA extraction using RLT mediated lysis of cells**

When isolating RNA from primary cells, such as Normal Human Epidermal Keratinocytes (NHEKs), Human Dermal Lymphatic Endothelial Cells (HDLECs), or Peripheral Blood Mononuclear Cells (PBMCs), RLT-mediated lysis was performed. Before RLT-mediated lysis of cells, 10µl beta-mercaptoethanol was added per 1ml of RLT buffer to ensure that any ribonucleases (RNases) released during lysis of the cell are denatured, and will therefore not degrade the RNA released by the cells. For adherent cells, such as NHEKs and HDLECs, the media the cells are grown in was removed, and the cells were washed with PBS. The PBS was then removed, and RLT buffer added directly to the cell monolayer. The lysate was then transferred to an RNase-free tube, and the lysate passed through a 20-gauge needle 6 times to ensure homogenization of the sample. The extracted RNA sample was then purified and concentrated using an RNeasy micro kit as described in section 2.2.6.

For PBMCs, the media containing the cells was transferred to an RNase-free Eppendorf tube. The tissue culture plate which contained the cells was then briefly washed with PBS to ensure any floating cells are picked up, and transferred to the eppendorf. These cells were then pelleted using a microfuge at 1200RPM for 5 minutes. During this time, RLT buffer is added directly to the tissue culture wells which previously contained the cultured cells. This was done as in our hands, even after washing the well with PBS, cells could still be found stuck to the tissue culture well. The lysed cells in the tissue culture well were then placed on ice. The supernatant was then removed from the centrifuged cells and discarded. The cell pellet was re-suspended, and the RLT buffer in the tissue culture wells added to its corresponding cell pellet. The cells were then homogenized by passing the lysate through a 20-gauge needle 6 times as before.

#### **2.2.5. RNA purification and concentration using an RNeasy mini kit**

The RNA from the full thickness biopsies was purified using the QIAGEN RNeasy mini kit as per manufacturer's instructions.

#### **2.2.6. RNA purification and concentration using an RNeasy micro kit**

RNA from primary cells (PBMCs, NHEKs, HDLECs) was purified using the QIAGEN RNeasy micro kit as per manufacturer's instructions.

#### **2.2.7. Reverse transcription (RT) converting RNA to cDNA**

The RNA was converted into cDNA using the Primer Design Precision Nanoscript Reverse Transcription kit as per manufacturer's instructions. In each RT reaction, an RT-negative control was added. Briefly, between 500ng to 1.5µg of RNA was used in the reaction depending on the source of the RNA. Full-thickness biopsies gave higher yields of RNA, and 1.5µg was used in the subsequent RT reactions. For adherent cells such as HDLECs and NHEKs, and for PBMCs, 500ng was used for each RT reaction.

#### **2.2.8. Polymerase chain reaction (PCR)**

The temperature of each stage in the PCR reaction used in this study was as follows; 95 degrees celcius for 3 minutes for initial denaturing of the DNA, then 35 cycles of the following, 95°C for 5 seconds (denaturing stage), 60°C for 20 seconds (annealing stage), and 72°C for 40 seconds (extension stage).

For the PCR reaction, Red PCR mastermix tubes (Roalab) were used. To these mastermix tubes, 1µl of a mix of forward and reverse primers for the gene of interest was added, in addition to 2µl of cDNA template, and 2µl of DNase and RNase free water. The primer sequences used are detailed in section 2.1.7.

#### **2.2.9. Quantitative polymerase chain reaction (QPCR)**

Although PCR is a useful technique for determining whether or not the gene of interest is present in a particular tissue, it cannot be used to accurately determine whether there is a difference in the expression of a particular gene between samples. QPCR allows the user to do just this, and as this study involved comparing expression levels of various genes between healthy controls, and diseased tissue, in addition to cytokine stimulations of various primary cells, QPCR was considered to be an important technique. In QPCR, a fluorescent dye called SYBR Green 1 is used which binds to double stranded DNA. Therefore, as the gene of interest is being amplified, the dye binds to nascent DNA

generating a fluorescent signal, and therefore the fluorescent signal increases. This signal can be measured quantitatively using a standard curve. The standard curve is generated by one of two methods. The first is the serial dilution of a plasmid in which the gene of interest has been inserted. Alternatively, a PCR can be carried-out for the gene of interest using the outer primers (See section 2.2.10.), on a sample where the gene of interest is expressed. The PCR product can then be purified, and concentrated as described in section 2.2.13, then used as a standard.

#### **2.2.10. Primer design**

For the QPCR reaction, two sets of primers for each gene being analysed were required. One set of primers, called the “inner primers”, are designed to bind to a small portion of the DNA sequence (usually between 80-150 base pairs in length). The second set of primers, or “outer primers”, are designed to bind to a portion of the DNA sequence that incorporates the sequence the inner primers bind to. The product generated from a PCR using the outer primers is used as the standard, enabling the generation of the standard curve for the gene of interest. The primers in this study were designed using the software Primer 3, which can be found on the following web-link. <http://frodo.wi.mit.edu/>.

The primers for use in QPCR have to be designed within certain specifications to ensure a successful reaction. The specifications for primers designed for this project are described below.

- Between 18 – 25 base pairs (bp) in length
- Between 45% – 60% GC content
- Primer melting temperature between 59 – 61°C
- Maximum self-complimentarity of 2
- Maximum 3' self-complimentarity of 1
- Primers binding to sections of DNA with 4 G or C bases are to be avoided.

Following these parameters allowed the successful design of primers for the genes of interest. The above conditions were the starting settings for each primer, but these settings could be relaxed if no specific primers were predicted using these initial conditions. The GC content could be relaxed from 45% - 60%, to 35% - 70%. Secondly, the Maximum self-complimentarity could be relaxed from 2 to 3. In addition, the primer's melting temperature can be reduced to 58°C. However, the 3' maximum self-complimentarity was not altered, and remained at 1. These are the specifications for the inner primers. For designing the outer primers, the initial start settings were the same, however, the size of the

product was increased in order to incorporate the PCR product generated by the inner primers.

#### **2.2.11. Confirming primer specificity**

To ensure the primers designed are specific for the gene of interest, two separate methods of confirming primer specificity were undertaken. First, the sequences of the forward and reverse primers were entered into the bioinformatics program, BLAST (web-link below).

<http://blast.ncbi.nlm.nih.gov/Blast.cgi>

Once the primers were confirmed to bind to the sequence of interest in BLAST, the primers were used in a PCR reaction. The PCR product was then electrophoresed on a 2% agarose gel containing ethidium bromide (intercalates with DNA and allows visualization) and visualized using the Alpha-imager detection system (section 2.2.12.). If the primers are specific, there should be one clear band visible. The band should correspond to the predicted size of the PCR product as calculated by Primer 3. The size is confirmed using hyperladder IV. The electrophoresed PCR product is then cut out of the 2% gel and purified using gel purification columns (section 2.2.13).

#### **2.2.12. Gel electrophoresis**

A 2% agarose gel was made by adding 1.6g of Agarose to 80mls of 1x tris-acetate EDTA (TAE) buffer. The agarose/TAE buffer mix was then microwaved until the agarose had dissolved. Upon cooling of the agarose gel, ethidium bromide was added to allow visualization of the PCR product. The gel was poured into the gel cassette with combs inserted, and left to cool until solid. The gels were run at 100 volts in 1xTAE buffer. An Alpha Imager gel documentation system was used for visualization of the PCR product under UV light.

#### **2.2.13. Developing standards for use in QPCR**

In the case of the assays for human D6, human GAPDH, human TATA binding protein (TBP), and mouse D6, the assays had already been developed in our laboratory. For these assays, a plasmid containing the gene of interest was used to generate the standard curve by serial dilution. For the rest of the QPCR assays in this study, the standard primers were used to generate a PCR product for the gene of interest. The PCR product was then run on a 2% agarose gel, and visualized using ethidium bromide as described in 2.2.12. If the band was the correct size, the band was excised from the gel using a scalpel, and the PCR product purified using the QIAquick Gel Extraction Kit as per manufacturer's instructions.



#### **2.2.14. Quantification of gene transcript numbers by QPCR**

QPCR allows the user to calculate the number of transcripts of a particular gene between different groups (e.g. control cohort and patient cohort). For this to be done, the number of transcripts of the gene of interest in the standard must be quantified. First, the concentration of DNA in each standard must be quantified. This is done using the optical density calculated using the nanodrop. The mass of the standard is then calculated using the following calculation;

OD x dilution factor x 50.

The following calculations are undertaken in order to calculate the number of transcripts for a particular gene. An exemplar calculation is given below using human CCL3 as an example:

CCL3 PCR product has a mass of  $5.07 \times 10^{-8}$  g/ $\mu$ l ( $1.4 \times 1 \times 50$ ) and a base pair size of 582. At first, the molecular weight of this PCR product was determined.

Average molecular weight for base paired nucleotide = 660 daltons

Molecular weight of double stranded DNA = 660 daltons x length of standard in bp

Molecular weight of double stranded DNA = 660 x 582

Molecular weight of double stranded DNA = 384,120 daltons

Number of moles of standard = mass in grams / Molecular weight of DNA

Number of moles of standard =  $5.07 \times 10^{-8}$  / 384,120 =  $1.319 \times 10^{-13}$  moles per  $\mu$ l

In order to determine the copy number from the number of moles, Avagadro's constant is used.

Copies DNA per  $\mu$ l = Avagadro's constant x moles per  $\mu$ l

Copies DNA per  $\mu$ l = ( $6.02 \times 10^{23}$ ) x ( $1.319 \times 10^{-13}$  moles per  $\mu$ l)

Copies DNA per  $\mu$ l =  $7.94 \times 10^{10}$  copies per  $\mu$ l

Standards of varying concentrations were used to obtain a standard curve ranged from  $1 \times 10^{-5}$ , to  $1 \times 10^{-9}$  dilutions of the original standard. Purified concentrated standard was first diluted to a concentration of  $1 \times 10^{-2}$  using 10mM TE buffer and stored for all future dilutions. Any further dilutions of the  $1 \times 10^{-2}$  standard used nuclease free water (Ambion).

#### **2.2.15. QPCR SYBR green protocol**

All samples were used in triplicate during the QPCR assay. A complete QPCR solution (called master-mix from here on) was made up immediately prior to preparing the samples

for QPCR. 20% more master-mix than required was made up to ensure that there would be enough if any errors in aliquoting the master-mix occurred. For each individual triplicate reaction the following was required;

- 0.15µl QPCR primer mix
- 4µl nuclease free water
- 5µl Power SYBR green mix

To each well 1µl of either cDNA sample, or cDNA standard was added to a 384-well reaction (Starlab) plate. After loading the samples, the plate was spun in a centrifuge for 10 seconds at 400RCF to ensure the cDNA was at the bottom of the plate. Then 9µl of QPCR master-mix was added to each well containing either cDNA sample, or cDNA standard. After loading the QPCR master-mix, the 384-well plate was briefly spun again in the centrifuge to ensure the samples were at the bottom of the plate. To ensure that any signals detected in the QPCR assay were not background signals, non-template water controls (NTCs) were added to the 384-well plate, in addition to the cDNA samples and standards.

#### **2.2.16. Normalising copy numbers of the gene of interest to suitable housekeeping genes**

To ensure that any changes in the expression of the genes of interest examined in this study were not simply due to variations in the amount RNA used in the RT reaction, housekeeping genes were used to normalize the transcript numbers of the gene of interest. The formula for calculating the absolute copy numbers, normalizing for the housekeeping gene is as follows;

- Copy number of gene of the interest/Copy number of the housekeeping gene  $\times 10^5$ .

The gene of interest is then expressed per 100,000 copies of the housekeeping gene. As a variety of tissues were used in this study, appropriate housekeeping genes were used for each of them. For human and mouse biopsies,  $\beta$ -actin was the housekeeping gene. For human PBMCs, TATA Binding Protein (TBP) was the housekeeping gene of choice. Finally for HDLECs and NHEKs, GAPDH was used as the housekeeping gene. Each of these housekeeping genes was individually validated, to ensure their appropriateness for the study.

#### **2.2.17. Cytospins**

Cytospins were carried out as follows. A cell suspension of no more than  $0.5 \times 10^6$  cells/ml was prepared. The slide for the cells to be spun onto was inserted into a metal holder, and

then a filter pad and cuvette attached. 200µl of cells was then added to the cuvette. It was then spun at 800rpm for 5 minutes. The slide was then removed from the metal holder, taking care not to disturb the cells on the slide through removal of the paper pad. The slides were left to dry, then fixed in 100% methanol for 20 minutes. The methanol was then removed, and the slide left to air dry. The sections were then either immuno-stained as described in section 2.2.20.2.1, or wrapped in foil, and placed in the minus 20°C freezer for long-term storage.

#### **2.2.18. Tissue culture**

##### **2.2.18.1 Culturing Normal Human Epidermal Keratinocytes (NHEKs)**

NHEKs were purchased from PromoCell, and were grown in T-25, or T-75 tissue culture flasks. The cells were cultured with Keratinocyte growth medium 2 from PromoCell, with gentamicin added. The media the cells were cultured in was replaced every second day with fresh media, and the cells cultured until approximately 90% confluent. Upon reaching 90% confluency, the cells are subcultured, and the cells used for experiments. Any remaining cells were frozen for later use, using the PromoCell freezing media (Cryo SFM).

##### **2.2.18.2. Culturing Human Dermal Lymphatic Endothelial Cells (HDLECs)**

As with NHEKs, HDLECs were purchased from PromoCell. The cells were cultured with Endothelial cell growth medium 2. Before transferring the cells into T-25 or T-75, the culture flask's plastic surfaces were incubated with 1x attachment factor (GIBCO) for at least 30 minutes in the incubator. In our hands, coating the cells with attachment factor increased the number of HDLECs attaching. The media the cells were cultured in was replaced every second day with fresh media, until approximately 90% confluent. At this point, the cells were either frozen down (using Cryo SFM), subcultured, or used in experiments.

##### **2.2.18.3. Isolating and culturing Peripheral Blood Mononuclear Cells (PBMCs)**

PBMCs used during this project were either from buffy coats (waste product from blood transfusions containing the white blood cells and platelets), or from blood samples from psoriasis patients, or healthy control volunteers. In both cases, the PBMCs were isolated through use of ficoll mediated density centrifugation. In brief, blood samples or buffy coats were diluted 1:1 with PBS/2mM EDTA, and layered in 8ml volumes onto 4mls of ficoll. The cells were then centrifuged for 25 minutes at 1700RPM at 12°C. After centrifugation, three layers should have formed. The top layer is the yellow coloured liquid, and is the

plasma. The middle white layer contain the PBMCs, and the lower red layer are the platelets (shown below).

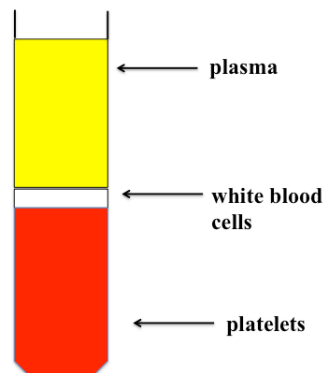


Figure 2.1. Schematic of ficoll separated buffy coats/ PBMCs

Using a pasteur pipette, the PBMCs were removed, taking care not to take up any of the plasma or platelets while doing so. The PBMCs were then washed by adding 4-5 volumes of PBS/2mM EDTA, and centrifuged at 1200RPM for 5 minutes at 12°C. This wash step was then repeated twice. The PBMCs were now purified.

#### **2.2.18.4. Stimulation of NHEKs, HDLECs and PBMCs**

All cytokines, and growth factors used in this study to stimulate the various cell populations were used at a concentration of 100ng/ml. Adherent cells (NHEKs and HDLECs), were grown until approximately 90% confluent. The media from these cells was then removed, and fresh media containing the cytokine or growth factor added to the cells. For PBMCs, the cells were seeded at the desired numbers, then the cytokine or growth factor added directly to the cells in the media.

#### **2.2.19. Laser Capture Microdissection (LCM)**

LCM was used to dissect the epidermis from psoriasis and healthy control tissue using the LCM microscope at the Beatson Institute for Cancer Research. Skin sections were initially cut onto RNase free slides (Leica) and stained using Mayers Haematoxylin by Colin Nixon (Histology manager) at the Beatson Institute for Cancer Research. The epidermis of the stained sections was then dissected from the dermis of the stained section using the Laser Capture Microscope at the Beatson Institute for Cancer Research. (Leica). The dissected epidermis would fall into an open Eppendorf tube directly below the site of dissection. The entire epidermis was then dissected from the sample, and then stored on dry ice. The RNA

from the dissected epidermis was then purified from the samples using the protocol outlined in section 2.2.6.

#### **2.2.20. Immunohistochemistry (IHC)**

Paraffin-embedded sections to be stained were initially de-waxed by two 10 minute incubations in xylene. This was followed by rehydration of the slides with two 5 minute incubations in 100% ethanol, one 5 minute incubation in 90% ethanol, and one 5 minute incubation in 70% ethanol. The cells were then washed with one 5 minute incubation in PBS-Tween 0.05% (PBS-T). The slides were then incubated in 1% Hydrogen Peroxide ( $H_2O_2$ ) for 30 minutes to block potential endogenous peroxidases. Two 5 minute washes in water were carried-out to remove the excess  $H_2O_2$ , followed by antigen retrieval.

Antigen retrieval is required to “unmask” the epitopes that the primary antibody binds to by removing the cross-linking of amino acids within the epitope.  
[http://www.rndsystems.com/literature\\_antigen\\_retrieval\\_methods.aspx](http://www.rndsystems.com/literature_antigen_retrieval_methods.aspx)

As several antibodies were used during this study, two separate antigen retrieval buffers were used. Citrate buffer (0.01M Citric acid, 0,05% tween, pH6) or Tris-EDTA (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0). Both Citrate buffer and Tris-EDTA were made up as per instructions on IHC world.  
[http://www.ihcworld.com/\\_protocols/epitope\\_retrieval/citrate\\_buffer.htm](http://www.ihcworld.com/_protocols/epitope_retrieval/citrate_buffer.htm)  
[http://www.ihcworld.com/\\_protocols/epitope\\_retrieval/tris\\_edta.htm](http://www.ihcworld.com/_protocols/epitope_retrieval/tris_edta.htm)

Citrate buffer was the antigen retrieval buffer of choice for the majority of primary antibodies detailed in section 2.1.1.1., with the exception of the D6 monoclonal antibody from R&D Systems. This antibody was found to work optimally using Tris-EDTA as the antigen retrieval buffer. In addition, the podoplanin antibody was found to work equally well using either Citrate buffer, or Tris-EDTA as the antigen retrieval buffer. The antigen retrieval buffer is heated in the microwave until boiling, then the sections are added and microwaved for another 6 minutes, and left to cool. After the antigen retrieval buffer had cooled, the sections were washed in water, then PBS-T for five minutes each. The areas around the tissue were then dried using blue roll, the sections encircled with a hydrophobic pen, and the sections incubated with serum block containing avidin blocker for 40 minutes. The sections were then washed briefly (20 seconds or less) to remove excess avidin blocker, and then the primary antibody was added to the sections, which also contained biotin blocker. The sections were then left to incubate overnight at 4°C. The next day, the sections returned to room temperature by leaving them on the bench for 30 minutes prior to

continuation of the protocol. Then 2 x 5 minute washes in PBS-T were performed, followed by incubation with the biotinylated secondary antibody for 30 minutes. The slides were then washed twice for 5 minutes in PBS-T. Depending on whether DAB staining, or IF staining was used to visualize the antigen, the protocol changes from this point on as detailed below.

#### **2.2.20.1. 3,3'-Diaminobenzidine (DAB) staining**

Once the biotinylated secondary antibody has been added to the stained sections, the Avidin/Biotinylated enzyme Complex (Elite Vectastain ABC kit) kit is prepared. To 2.5ml PBS-T one drop of solution A, and one drop of solution B is added. After a 30 minute incubation of the biotinylated secondary antibody, two 5 minute washes with PBS-T were performed, followed by a 30 minute incubation of the sections with the ABC kit, followed by two 5 minute washes in PBS-T. Just prior to the two 5 minute washes, DAB substrate was made up as per manufacturer's instructions (one drop DAB in 1ml PBS-T). The DAB was then added to the sections, and left for 1 minute. The reaction is stopped by immersing the slide in water. The slides were then washed in PBS-T for 5 minutes. The sections were then stained with Mayer's Haematoxylin by dipping the slides in twice, then washed in water to remove excess stain. The slides were then dehydrated by immersing them in increasing concentrations of ethanol in the following order; 70% ethanol 2 minutes x2, 90% ethanol 2 minutes x2 and 100% ethanol 2 minutes x2. Then the slides were "cleared" of any excess water by immersing them in xylene 3 times for 2 minutes each. The sections were then fixed with DPX.

#### **2.2.20.2. Immunofluorescent (IF) staining**

After a 30 minute incubation of the biotinylated secondary antibody, 2 x 5 minute washes in PBS-T are performed. Fluorochromes conjugated to avidin were diluted (1 in 500 dilution) in PBS pH8. The tissue sections were then incubated with the fluorochromes for 40 minutes. Fluorescein isothiocyanate (FITC) conjugated to avidin was the IF molecule of choice for D6 in this study. Other IF molecules conjugated to avidin were used to co-stain various specific populations of cells, including Texas Red, and Cy5. The cells were then washed twice for 5 minutes to remove excess fluorochromes. The slides were then mounted using Vectamount containing DAPI which binds to the nucleus and is visualized using IF. The edges of the sections were then sealed with clear nail varnish.

#### **2.2.20.2.1. IF staining of primary cells**

The media the cells were cultured in was removed, the cells washed in PBS, then fixed in 100% methanol for 20 minutes. After fixation, the methanol was removed, and the cells allowed to air dry. Once dry, the cells were incubated with serum block, then primary antibody added, and left overnight. The next day, the slides were warmed to room temperature, washed in PBS-T, then incubated with secondary antibody for 30 minutes. The cells were then washed using PBS-T, and FITC conjugated to avidin added, and incubated for 40 minutes. The cells were washed again in PBS-T, then fixed using Vectashield containing DAPI. The slides were then examined for IF using the Zeiss Axiovision Imaging Microscope. For cytopun cells, after fixation with 100% methanol, the sections were left to air dry. Then the cells were stained as detailed above.

#### **2.2.20.2.2. IF co-staining**

For co-staining with two separate antibodies, the protocol is as outlined in section 2.2.20. and 2.2.20.2. with minor changes. Specifically, after the fluorochrome has been incubated with the section for 40 minutes and washed twice in PBS-T (2.2.20.2.), instead of sealing the slide, the following should be performed.

The sections are incubated with serum block containing avidin blocker for 40 minutes. The sections were then washed briefly (20 seconds or less) to remove excess avidin blocker, and then the primary antibody was added to the sections, which also contained biotin blocker. The sections were then left to incubate overnight at 4°C. The next day, the sections returned to room temperature by leaving them on the bench for 30 minutes prior to continuation of the protocol. Then 2 x 5 minute washes in PBS-T were performed, followed by incubation with the biotinylated secondary antibody for 30 minutes. The slides were then washed twice for 5 minutes in PBS-T. The slides were then fixed using Vectamount containing DAPI which binds to the nucleus and is visualized using IF. The edges of the sections were then sealed with clear nail varnish.

#### **2.2.20.3. Antibody concentrations**

The combination of primary and secondary antibodies, in addition to isotype controls or animal IgG fractions used in this study as appropriate, are as follows,

	<b><u>Concentration</u></b>
Rat anti-human D6 monoclonal antibody	3µg/ml
Rat IgG2a isotype control	3µg/ml
Biotinylated anti-rat IgG secondary antibody, raised in rabbit	7.5µg/ml

	<b><u>Concentration</u></b>
Rabbit anti-human D6 polyclonal antibody	0.5µg/ml
Rabbit IgG fraction control	0.5µg/ml
Biotinylated anti-rabbit IgG secondary antibody, raised in goat	7.5µg/ml
Rabbit anti-human Podoplanin polyclonal antibody	0.5µg/ml
Rabbit IgG fraction control	0.5µg/ml
Biotinylated anti-rabbit IgG secondary antibody, raised in goat	7.5µg/ml
Mouse anti-human CCL2 monoclonal antibody	5µg/ml
Mouse IgG2b isotype control	5µg/ml
Biotinylated anti-mouse IgG secondary antibody, raised in horse	7.5µg/ml
Goat anti-human CCL5 polyclonal antibody	5µg/ml
Goat IgG control	5µg/ml
Biotinylated anti-goat IgG secondary antibody, raised in rabbit	7.5µg/ml
Mouse anti-human CD3 monoclonal antibody	1µg/ml
Mouse IgG1 isotype control	1µg/ml
Biotinylated anti-mouse IgG secondary antibody, raised in horse	7.5µg/ml
Mouse anti-human CD68 monoclonal antibody	1µg/ml
Mouse IgG2a isotype control	1µg/ml
Biotinylated anti-mouse IgG secondary antibody, raised in horse	7.5µg/ml
Goat anti-human DLEC/BDCA-2 polyclonal antibody	0.25µg/ml
Goat IgG control	0.25µg/ml
Biotinylated anti-goat IgG secondary antibody, raised in rabbit	7.5µg/ml
Mouse anti-human Tryptase monoclonal antibody	0.43µg/ml
Mouse IgG1 isotype control	0.43µg/ml
Biotinylated anti-mouse IgG secondary antibody, raised in horse	7.5µg/ml
Mouse anti-human CD45 monoclonal antibody	1µg/ml
Mouse IgG1 isotype control	1µg/ml



**2.2.21. 30plex luminex kit**

The multiplex kit was obtained from Invitrogen, and was used to measure 30 different inflammatory mediators in a large number of samples. 10 healthy controls and 56 psoriasis plasma samples were investigated as per manufacturer's instructions with one exception. The beads and biotinylated antibodies used in the 30plex were diluted 1 in 2 in the buffers provided, and divided over two plates, to allow a greater number of samples to be simultaneously analysed.

**2.2.22. Imiquimod mouse model of psoriasis**

All mice used were 10 weeks just prior to the start of experimentation. The day prior to treatment with Imiquimod, the dorsal skin, and the neck skin of female C57BL/6 (Harlan, UK) mice was shaved. The following day, one third of a sachet of Imiquimod, or Vaseline control, was applied to the shaved dorsal skin of the mouse. This was done once a day for 5 days. On day 6, the mice were culled by an overdose of CO<sub>2</sub>, and the dorsal skin treated with Imiquimod, or Vaseline control was removed using scissors. In addition, neck skin from the mice treated with Imiquimod or Vaseline control was also obtained. The tissue was then cut in half, and half placed in RNA later for analysis using QPCR, and half placed in formalin for fixation and IHC. All handling of mice, application of Imiquimod or Vaseline, and the culling of mice was performed by Dr Jenny Lee (Professor Graham's laboratory) under Professor Gerard Graham's home office license. Once the mice had been sacrificed, and the samples put in RNA later or formalin, the analysis of these samples was performed by Mark Singh.

**2.2.23. Micro-trauma of human skin**

Patient volunteers were subjected to tape stripping. This involved sticking scotch tape to a patient's uninvolved psoriatic skin, and pulling it off to induce local inflammation. This was repeated an additional 9 times. The next day, a full thickness biopsy was taken from the patient at the site of tape stripping, and a contralateral uninvolved psoriatic skin site. The biopsy was then cut into two, and half placed in RNA later for analysis using QPCR, and half put in formalin for fixation and embedding for use in IHC.

**2.2.24. Statistical analysis**

The statistical tests used for each experiment are found in the figure legends. Statistical analysis was performed using the software GraphPad Prism.

#### **2.2.25. Microscopy**

Imaging analysis was performed using either the Axiovert S100 or Axiovision (Ax 10) Imager M2 from Zeiss.

#### **2.2.26. PBMC-D6 cytospin counts, *in situ* leukocyte-D6 counts, NHEK-D6 or HDLEC-D6 counts, and epidermal T-cell counts**

For each set of counts in this study, 10 random images were taken of the image of interest, and the number of cells of interest counted. The average value was then calculated and graphed.

#### **2.2.27. Lipid depletion**

Lipid Removal Agent (LRA) was obtained from Sigma. LRA was then added to 10% human serum at a concentration of 0.1g/ml, and left to incubate for 2 hours while continuously spinning. After the 2 hour incubation, the LRA was removed by centrifuging the serum/LRA mix for 10 minutes at 2000RPM. The 10% human serum was then removed from the pelleted LRA. Human PBMCs were then incubated with either lipid depleted 10% human serum, or 10% human serum without prior incubation with LRA.

#### **2.2.28. Information on patient samples**

No additional information e.g. sex, age, treatment, PASI score, treatments the patients are currently receiving, was analysed for the patients who donated a biopsy for analysis of D6 expression by QPCR and IHC. In addition, no patient information was available for the analysis of PBMC-D6 expression displayed in Figures 3.29. and 3.33. In hindsight, this information is of great importance, and of course would have been analysed if the experiment was performed again. Each of these parameters could have been used to further dissect D6 expression in psoriasis. For example, dividing the patients into male and female for analysis could have been used to determine if any differences in D6 expression were found in psoriasis patients of different gender. In addition, age would have been very interesting to examine, as the immune response wanes as an individual becomes older. It would be interesting to see if D6 expression in psoriasis patients decreases with age, as the immune response weakens over time. From other studies performed in the group, it appears that patients on treatments for psoriasis actually increase D6 expression as the disease goes into remission, therefore the highest D6 expression detected in some of the patients could be due to this. Similarly, PASI score would have been useful as it would allow an analysis of D6 expression against a marker of disease, although this is analysed later in a second cohort of patients. Therefore, the initial analysis of D6 expression, while

interesting, could be masking very important information which has been ‘hidden’ by simply putting the data sets together.

For the second cohort of patients used to analyse PBMC-D6 expression (First analysed in Figure 4.10.), some additional information was obtained, and is listed below;

- Gender of participants, 30 male, 24 female
- Age range, 23 years old – 72 years old (median – 48, mean – 43.6)
- PASI score, 0 – 26

While the PASI score was used to analyse whether D6-PBMC expression relates to disease severity, the additional parameters listed above could have been used to analyse D6 expression in a variety of other contexts, such as differences in gender, and age, as discussed previously.

## **Chapter 3 – D6 in psoriasis**

### 3. D6 in psoriasis

#### 3.1. Introduction

Psoriasis is a chronic inflammatory skin disorder that affects approximately 2% of the caucasian population world wide (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). It is characterized by the appearance of red scaly plaques on the patient's skin. These plaques can occur anywhere on the patient, but tend to be at sites that are prone to micro-trauma (e.g. elbows, knees and scalp). At the microscopic level, psoriasis is characterized by hyperproliferation of the epidermis, in addition to infiltration of leukocytes into the lesion, and an uncoupling of the differentiation of the epidermal keratinocytes (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). It is believed that an effector T-cell response drives disease pathology in psoriasis. Specifically, Th1 T-cells and Th17 T-cells, in addition to Th22 cells are known to be involved. Although the trigger for lesion development is currently unknown, there are data suggesting that various triggers such as trauma to uninvolved psoriatic skin can initiate lesion formation (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012).

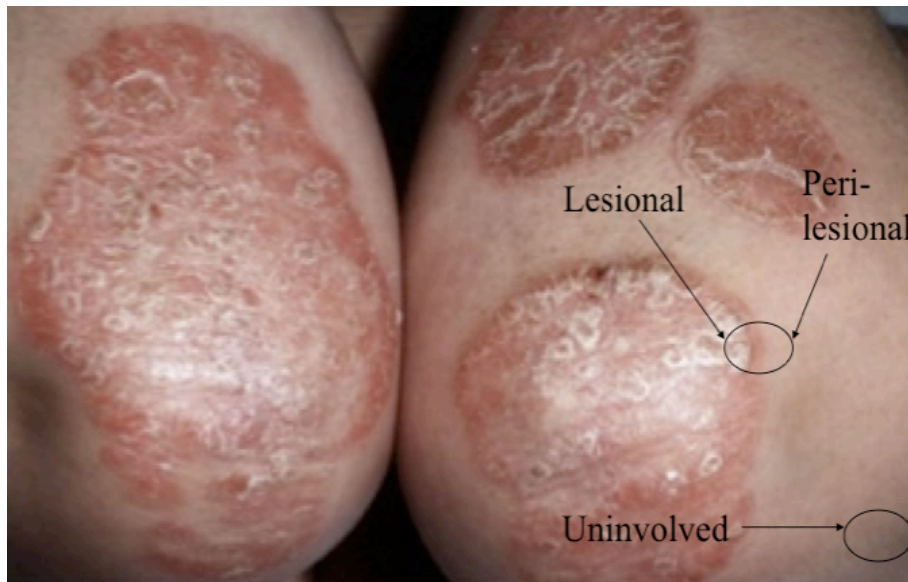
As discussed in the introduction, D6 is a chemokine-scavenging receptor that can bind and internalize at least 14 inflammatory CC chemokines *in vitro* (Graham, 2009). D6 KO mice treated with phorbol ester have been shown to develop a psoriasiform pathology (Jamieson et al., 2005). With these initial data, it was hypothesized that,

‘ a loss of D6 expression in uninvolved psoriatic skin is associated with the development of psoriatic lesions’.

The aim of the work presented in this chapter was to test this hypothesis. Therefore, biopsies were obtained from psoriasis patients and healthy controls. Two types of biopsies were taken from psoriasis patients as shown in Figure 3.1. The first type of biopsy was an elliptical biopsy, which covered lesional psoriatic tissue, and the adjacent peri-lesional tissue (Figure 3.1.). The second type of biopsy was taken from a site distant from any lesions (greater than 8cm from any visible lesions), and was classified as ‘uninvolved’ psoriatic skin (Figure 3.1.). To examine Peripheral Blood Mononuclear Cell (PBMC) D6 expression, a blood sample was taken from psoriatic patients and healthy controls. D6 expression was examined using QPCR, in addition to antibody staining to analyse the expression of D6 protein. To phenotype D6 positive cells *in situ*, D6 staining antibodies were used in conjunction with staining antibodies for a variety of leukocyte and LV markers.

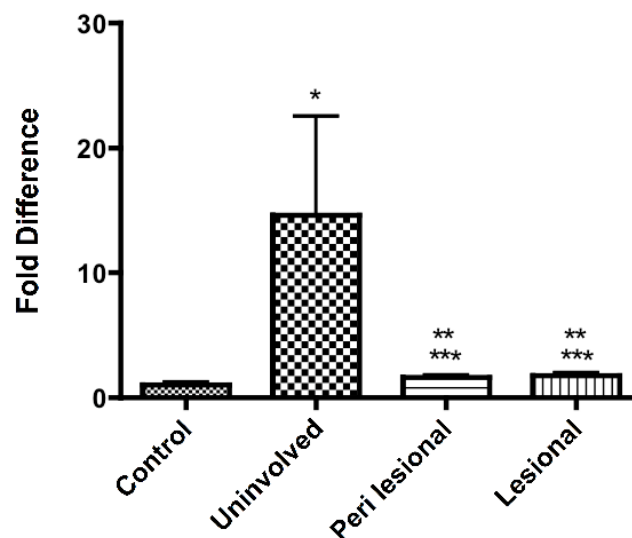
### **3.2. Biopsy collection and analysis of D6 expression**

D6 expression in skin samples was first examined using QPCR. The level of D6 expression in the healthy controls was set to '1' (Figure 3.2.), and D6 expression in the various psoriasis tissues calculated as a fold change relative to the healthy controls (Figure 3.2.). A significant elevation of D6 transcript levels between healthy control and uninvolved psoriatic skin was observed as denoted by \* (Figure 3.2.). In addition there is a significant reduction of D6 expression comparing uninvolved psoriatic skin to peri-lesional psoriatic skin, and uninvolved psoriatic skin to lesional psoriatic skin as denoted by \*\* (Figure 3.2.). Finally there is a modest elevation in D6 transcript numbers between control and peri-lesional psoriatic skin, and control and lesional psoriatic skin that reaches statistical significance, denoted by \*\*\* and shown in Figure 3.2. Thus, D6 is elevated in psoriasis tissue compared to healthy controls. However, the significant increase in D6 expression in uninvolved psoriatic skin, compared with control skin, or peri-lesional, or lesional psoriatic skin, indicates that D6 may be particularly important at this site.



**Figure 3.1. Visual description of the sites of biopsy.**

Biopsies were taken from psoriasis patients either from histologically normal, uninvolved psoriatic skin, or an elliptical biopsy taken encompassing both peri-lesional and lesional skin.



**Figure 3.2. D6 RNA expression in healthy controls and psoriasis.**

Levels of D6 transcripts were examined in healthy controls and psoriasis tissue. Healthy control D6 expression levels were set to '1', and D6 expression in psoriasis tissue calculated with reference to the healthy controls. D6 expression was normalized to the housekeeping gene  $\beta$ -actin. Healthy control n=10, uninvolved psoriatic skin n=5, peri-lesional psoriatic skin n=16 and lesional psoriatic tissue n=16. \* denotes comparing levels of D6 expression between healthy control tissue to uninvolved psoriatic tissue. \*\* denotes comparing levels of D6 expression between uninvolved psoriatic tissue and peri-lesional and lesional tissue. \*\*\* denotes comparing levels of D6 expression between healthy controls and peri-lesional psoriatic tissue and healthy controls and lesional psoriatic tissue. Statistical significance was determined using a Kruskal Wallis Test and Dunn's Multiple Comparison Test.

### **3.3. D6 antibody staining methodology**

Commercial antibodies were obtained in order to examine D6 protein expression *in vivo*. D6 monoclonal (R&D Systems) and D6 polyclonal (Sigma) antibodies were obtained, and placental sections used to optimize the staining protocol. The placenta was selected, as this is an established site of D6 expression (Madigan et al., 2010, Martinez de la Torre et al., 2007, Nibbs et al., 1997b).

#### **3.3.1. R&D Systems D6 monoclonal antibody immuno-staining on placental sections**

The monoclonal D6 antibody from R&D Systems was tested first. As shown in Figure 3.3.A., D6 immuno-reactivity was detected in the placental sections, localizing to the syncytiotrophoblast layer when incubated with the D6 monoclonal antibody. In addition, no immuno-staining was detected in placenta sections incubated with Rat IgG2a isotype control (Figure 3.3.B.), suggesting that the immuno-stains were specific for D6.

#### **3.3.2. Sigma D6 polyclonal antibody immuno-staining on placental sections**

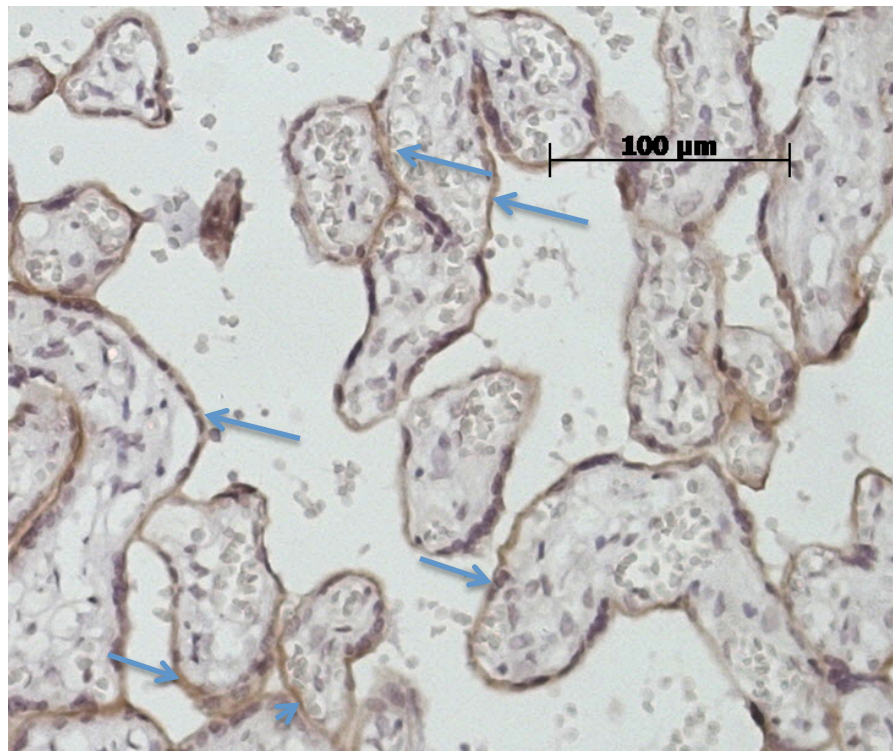
Next the Sigma D6 polyclonal antibody was tested. D6 immuno-staining was detected on the syncytiotrophoblast of the placenta in sections incubated with the Sigma D6 polyclonal antibody (Figure 3.4.A.). Placenta sections incubated with rabbit IgG fractions were not immuno-reactive as shown in Figure 3.4.B. These data suggest the D6 Sigma polyclonal antibody stains specifically for placenta D6.

#### **3.3.3. R&D Systems D6 monoclonal antibody immuno-staining on LVs**

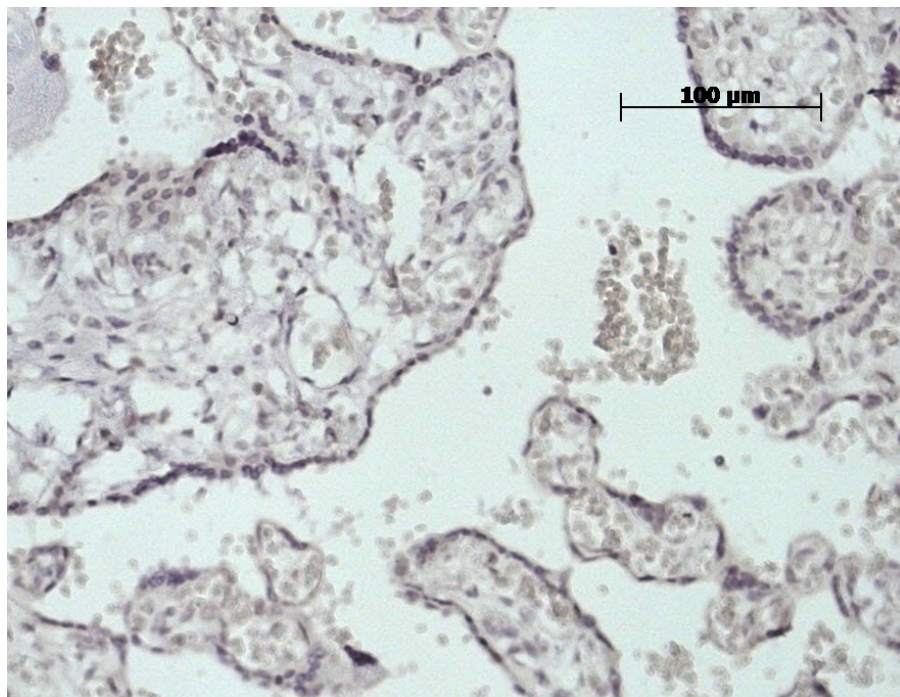
As a secondary test of specificity, LV staining was studied as this is another established site of D6 expression (Nibbs et al., 2001). D6 staining has been found on some, but not all LVs in healthy control skin (Nibbs et al., 2001). Therefore, human skin sections were used to test whether the monoclonal (R&D Systems) and polyclonal (Sigma) D6 antibodies were also immuno-reactive for subsets of LVs.

As shown in Figure 3.5.A., human skin sections incubated with the R&D Systems D6 monoclonal antibody had detectable immuno-reactivity, localizing to the LVs (highlighted by a blue arrow). No immuno-reactivity was detected in human skin sections incubated with Rat IgG2a isotype control (Figure 3.5.B.), suggesting the immuno-reactivity observed in human skin sections incubated with the monoclonal antibody was specific for D6.





A

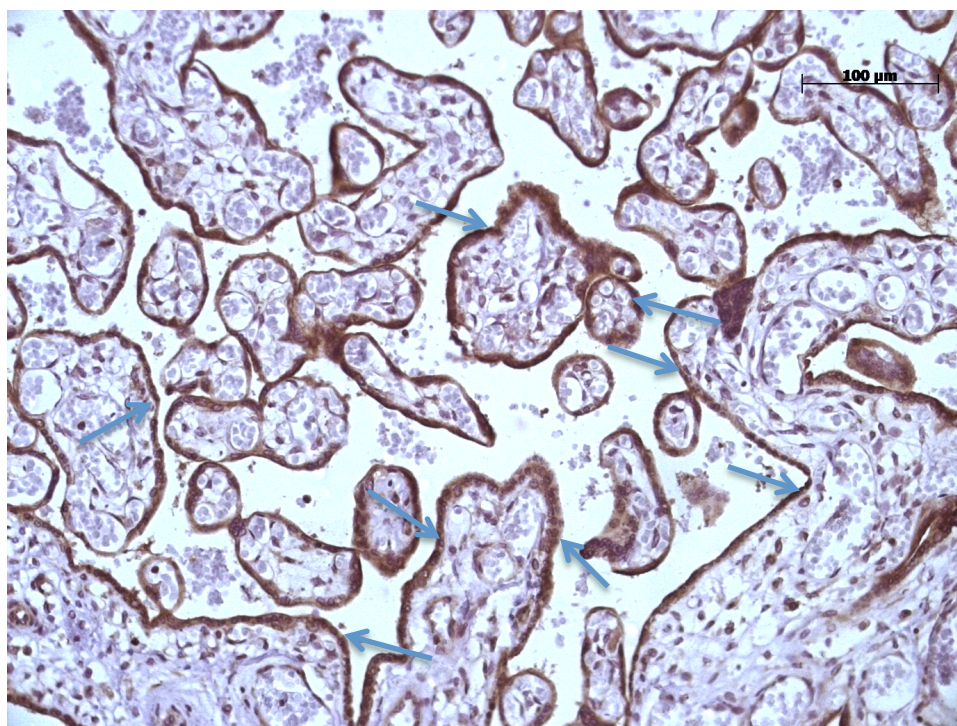


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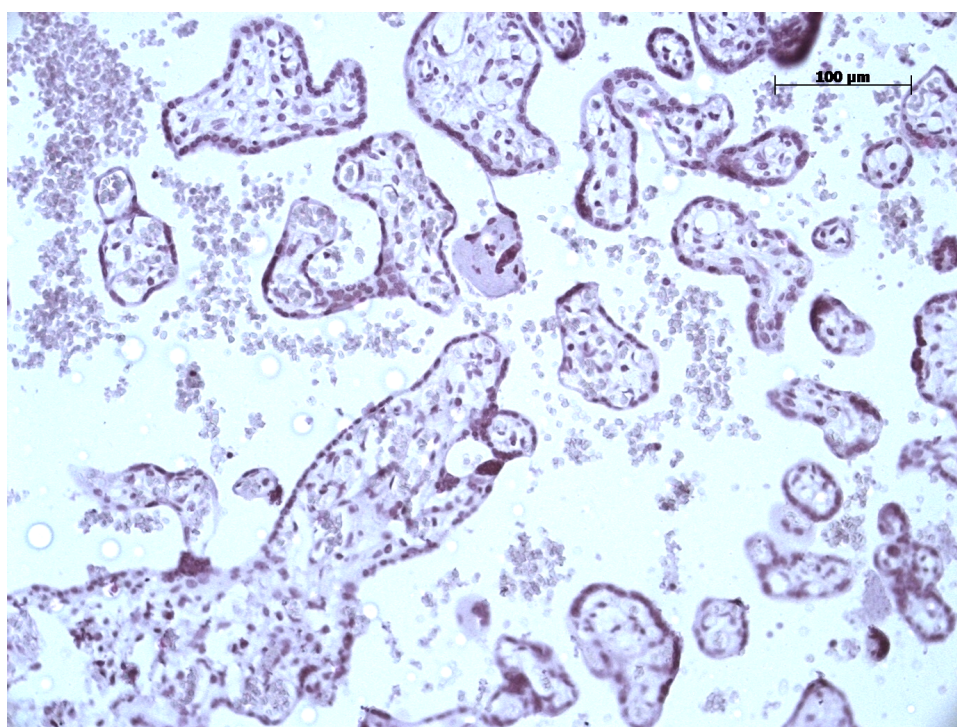
**Figure 3.3. R&D Systems D6 monoclonal antibody immuno-reactivity in human placenta.**

Human placental sections were incubated with either A. R&D Systems D6 monoclonal antibody (1.67μg/ml), (D6 Immuno-reactivity highlighted by blue arrows), or B. Rat IgG2a Isotype control (R&D Systems) (1.67μg/ml). Scale bars 100μm. Images are representative of 5 different placental sections tested. Sections were counterstained with Haematoxylin and Eosin (H&E).





A

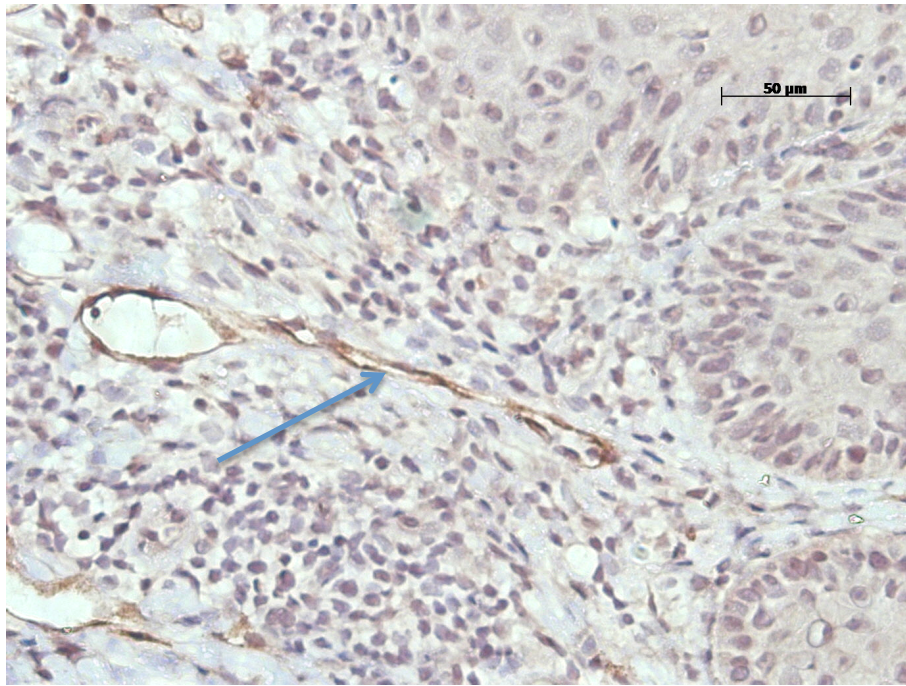


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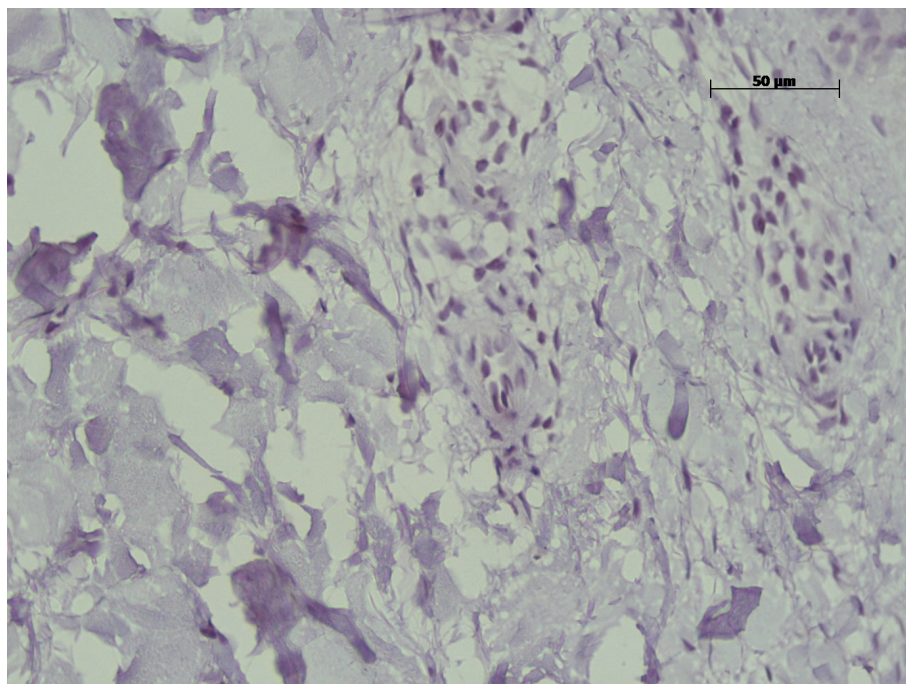
**Figure 3.4. Sigma D6 polyclonal antibody immuno-reactivity in human placenta.**

Human placental sections were incubated with either A. Sigma D6 polyclonal antibody (2.2 $\mu$ g/ml) (D6 Immuno-reactivity highlighted by blue arrows), or B. Rabbit IgG fractions (DAKO) (2.2 $\mu$ g/ml). Scale bars 100 $\mu$ m. Images are representative of 5 different placental sections tested. Sections were counterstained with H&E.



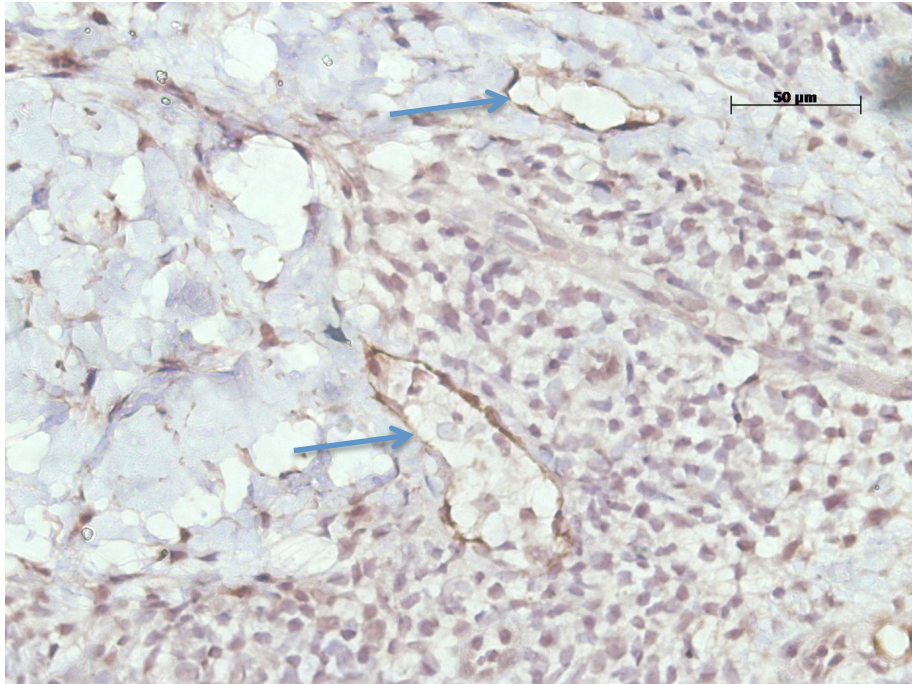


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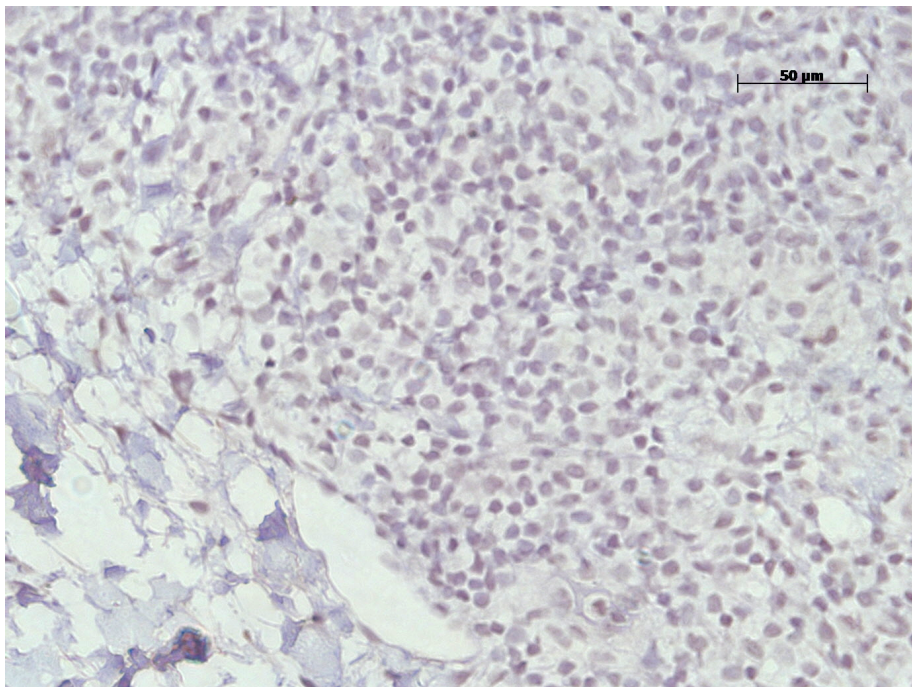


B

**Figure 3.5. R&D Systems D6 monoclonal antibody immuno-reactivity in human skin.** Human skin sections were incubated with either A. R&D Systems D6 monoclonal antibody (1.67 $\mu$ g/ml), (D6 immuno-reactivity localizing to a LV is highlighted by a blue arrow), or B. Rat IgG2a Isotype control (R&D Systems) (1.67 $\mu$ g/ml). Scale bars 50 $\mu$ m. Images are representative of 5 different human skin sections tested. Sections were counterstained with H&E.



A



B

**Figure 3.6. Sigma D6 polyclonal antibody immuno-reactivity in human skin.**

Human skin sections were incubated with either A. Sigma D6 polyclonal antibody (2.2 $\mu$ g/ml), (D6 immuno-reactivity localizing to LVs is highlighted by a blue arrow), or B. Rabbit IgG fractions (DAKO) (2.2 $\mu$ g/ml). Scale bars 50 $\mu$ m. Images are representative of 5 different human skin sections tested. Sections were counterstained with H&E.

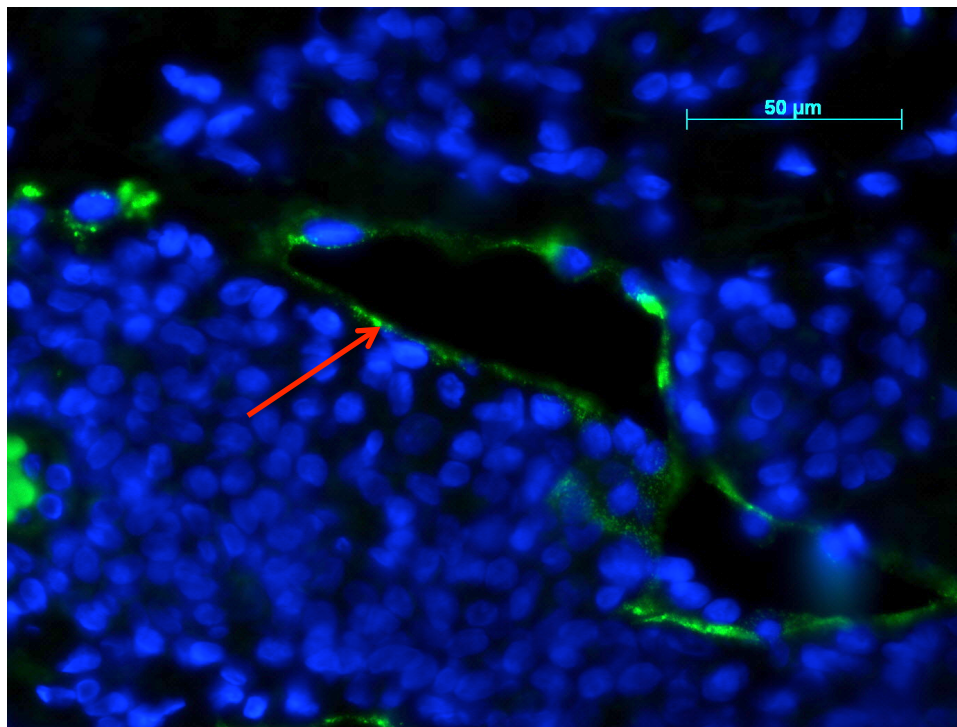


#### **3.3.4. Sigma D6 polyclonal antibody immuno-staining on LVs**

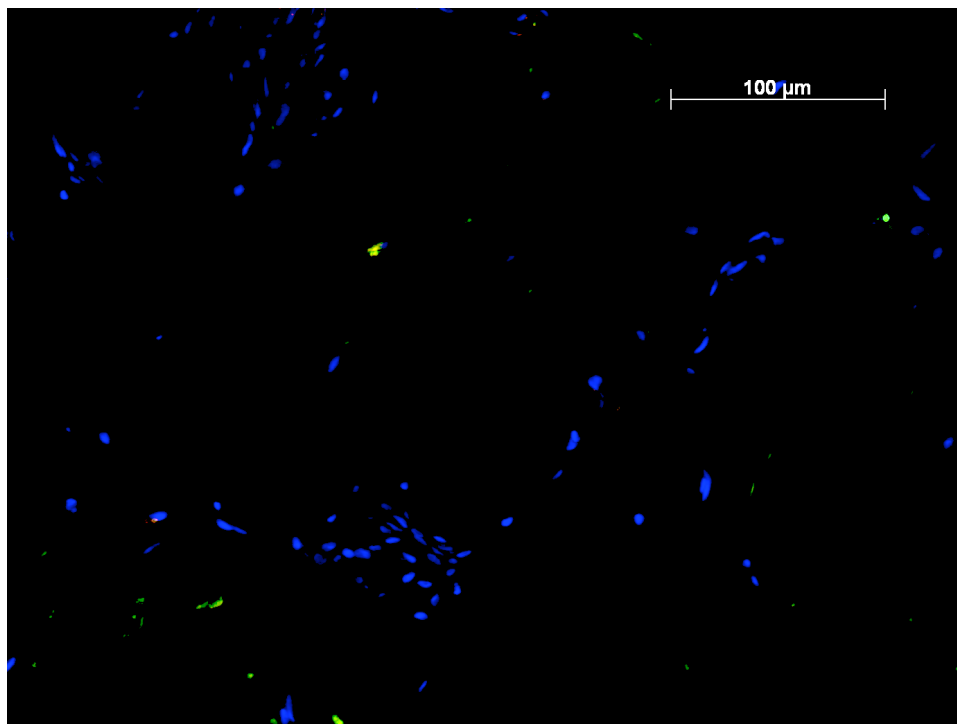
Similar to the R&D Systems D6 monoclonal antibody, the Sigma D6 polyclonal antibody was found to detect D6 immuno-reactivity on LVs as shown in Figure 3.6.A. No background staining was detected in human skin sections incubated with Rabbit IgG fractions (Figure 3.6.B.).

#### **3.3.5. Use of Immunofluorescence for antigen visualisation**

Two D6 staining antibodies had now been confirmed to detect immuno-reactivity at established sites of D6 expression, i.e. the LVs (Nibbs et al., 2001), and the placenta (Madigan et al., 2010, Martinez de la Torre et al., 2007, Nibbs et al., 1997b). However, use of DAB staining as the method of detection has the disadvantage of only allowing the visualisation of one antigen of interest. In order to perform co-staining with additional cellular markers, the detection system used was changed from DAB staining, to use of immunofluorescence (IF) as the detection system. This allows the user to perform immuno-staining using several different antibodies that can be detected using a variety of IF molecules that are detectable at different wavelengths. Therefore, the protocols for both D6 staining antibodies were modified to allow the use of an IF molecule after incubation with the biotinylated secondary antibody. This IF molecule was conjugated to an avidin molecule, thus ensuring strong binding between the IF molecule and the secondary antibody. Both the D6 monoclonal antibody (R&D Systems) (Figure 3.7.A.), and the D6 polyclonal antibody (Sigma) (Figure 3.8.A.) could be used to successfully detect D6 immuno-reactivity in LVs in human skin. No detectable immuno-reactivity was detected in human skin sections incubated with Rat IgG2a isotype control (Figure 3.7.B.) or Rabbit IgG fractions (Figure 3.8.B.). Thus, IF is suitable for D6 immuno-staining using both antibodies.



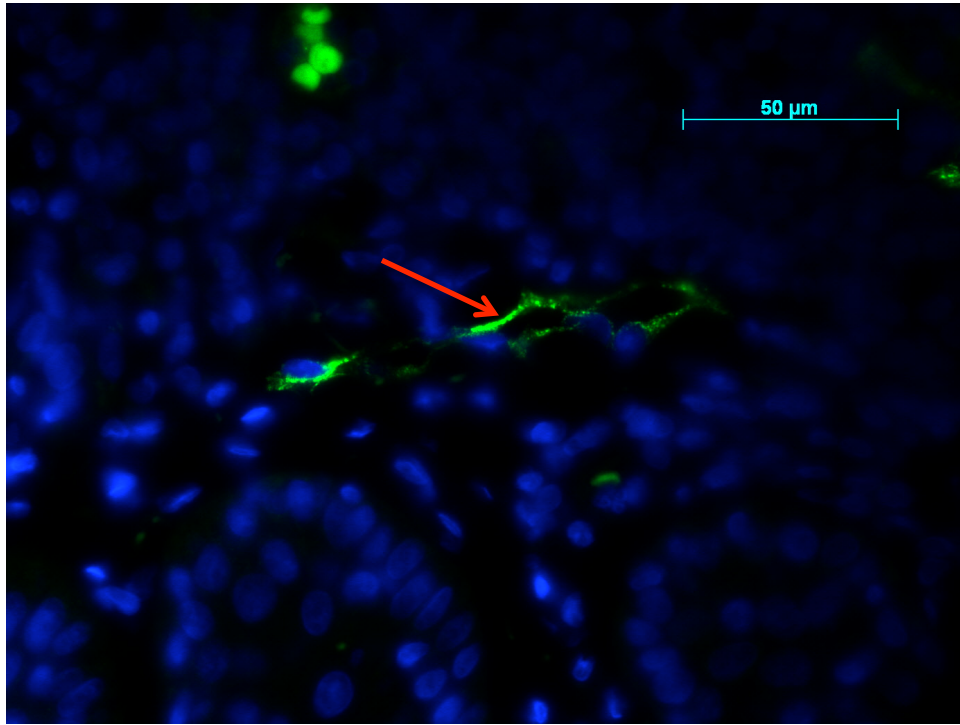
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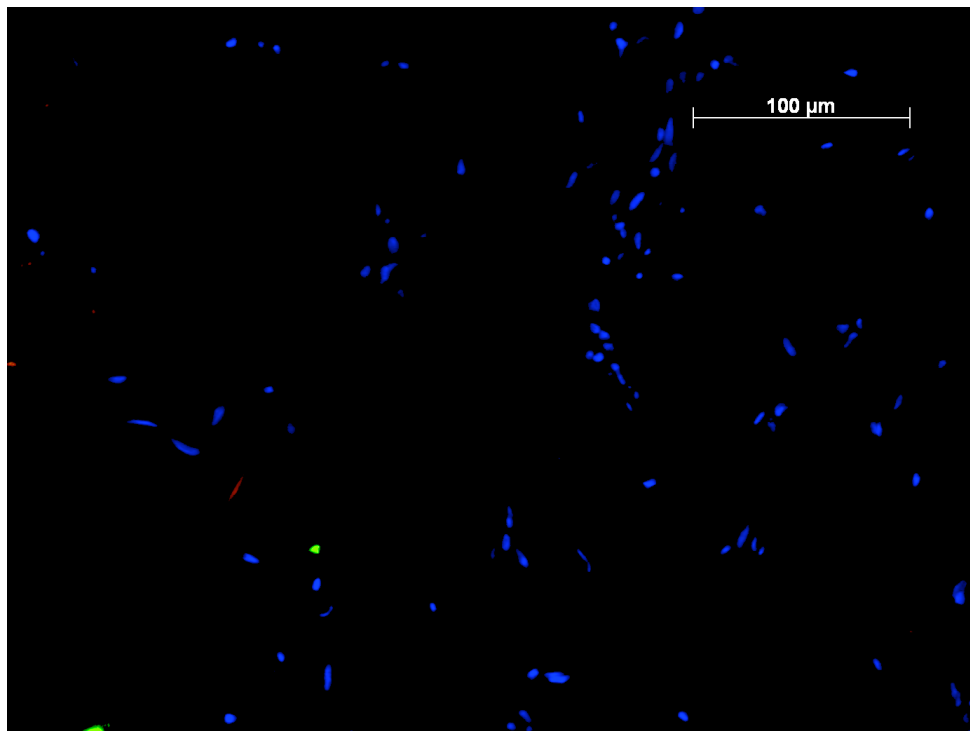
B

**Figure 3.7. R&D Systems monoclonal antibody stained LVs.**

A. R&D Systems D6 monoclonal antibody stained LV (FITC) in human skin or B. Rat IgG2a (R&D Systems). D6 antibody and Rat IgG2a were used at 3μg/ml. Scale bar A = 50μm, Scale bar B = 100μm. Images are representative of 5 different human skin sections tested. Nuclei were visualised using DAPI.



A



B

**Figure 3.8. Sigma D6 polyclonal antibody stained LVs**

A. D6 (Sigma) polyclonal stained LV (FITC) in human skin or B. Rabbit IgG fractions (DAKO). D6 antibody and Rabbit IgG fractions were used at 0.5μg/ml. Scale bar A = 50μm, scale bar B = 100μm. Images are representative of 5 different human skin sections tested. Nuclei were visualised using DAPI.

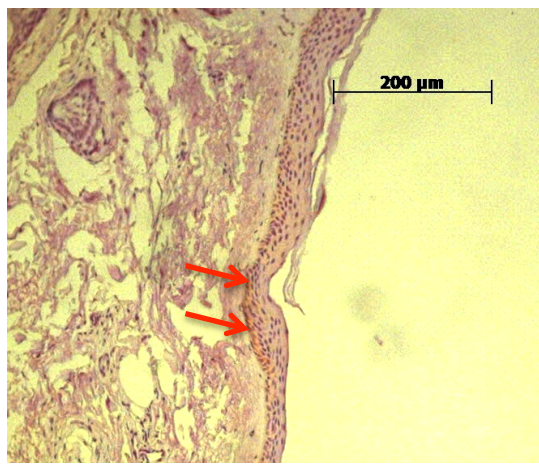
### **3.4. D6 expression in healthy controls and psoriasis.**

#### **3.4.1. Detection of D6 expression using antibody staining**

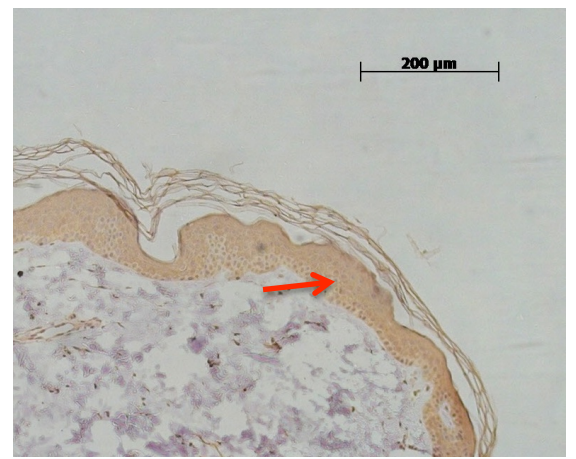
With the monoclonal and polyclonal D6 staining antibodies tested and validated, D6 expression was analysed in healthy control and psoriasis tissue to see if it agreed with the PCR results. Interestingly, D6 immuno-reactivity was observed on the epidermis of healthy control skin, in addition to the epidermis of uninvolved, peri lesional and lesional skin from psoriasis patients, indicated by red arrows (Figures 3.9.-3.10.). Using the D6 (R&D Systems) monoclonal and D6 (Sigma) polyclonal antibodies, it was observed that D6 expression in healthy controls was predominantly restricted to the lower levels of the epidermis (Figures 3.9.A. and 3.10.A.). In uninvolved psoriatic skin (Figures 3.9.B. and 3.10.B.), D6 expression was found throughout the epidermis. In peri-lesional skin, D6 expression was again localised to the lower levels of the epidermis (Figures 3.9.C. and 3.10.C.). It was noted that in peri-lesional skin, D6 staining was more uniform compared to healthy controls. In healthy controls, sections of the stratum basale, (the lowest levels of the epidermis, adjacent to the dermis) were D6-positive, and sections of the stratum basale were D6-negative (Figure 3.9.A and Figure 3.10.A). In lesional psoriatic skin, D6 is found throughout the viable epidermis (Figure 3.9.D. and 3.10.D.). In addition to epidermal D6 immuno-reactivity in psoriatic lesional sections, leukocyte-like cells were stained D6 positive in the dermis of psoriasis lesional sections (Figure 3.9.D. and 3.10.D., denoted by yellow arrow). These D6 positive, leukocyte-like cells are examined in section 3.7. Human skin sections incubated with either Rat IgG2a (Figure 3.9.E.), or Rabbit IgG fractions (Figure 3.10.E.) did not have any detectable immuno-reactivity.

To improve the quality of the stain, healthy control and the various psoriasis tissues were stained for D6 and IF used as the method of detection. As shown in Figures 3.11. and 3.12., the staining patterns observed using IF staining matched the staining pattern of D6 observed using DAB staining. Specifically, in healthy controls, D6 immuno-reactivity was sparse, and confined exclusively to the stratum basale (Figures 3.11.A. and 3.12.A.). Uninvolved psoriatic skin was D6-positive throughout the viable layers of the epidermis (Figures 3.11.B. and 3.12.B.). In peri-lesional skin, the lower layers of the epidermis were D6 positive (Figure 3.11.C. and 3.12.C.). In lesional psoriatic skin, the entire epidermis was D6 positive (Figures 3.11.D. and 3.12.D.).

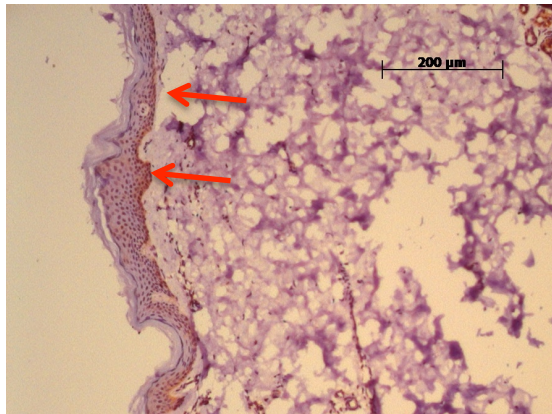




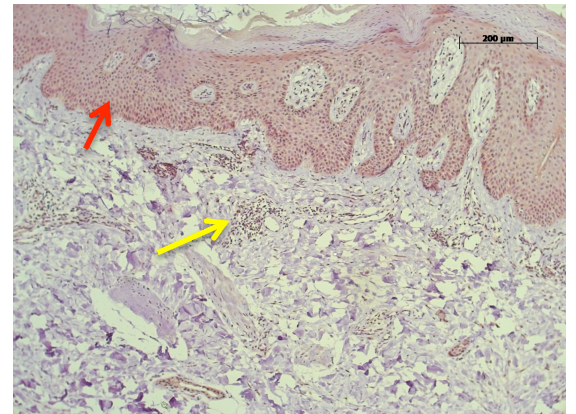
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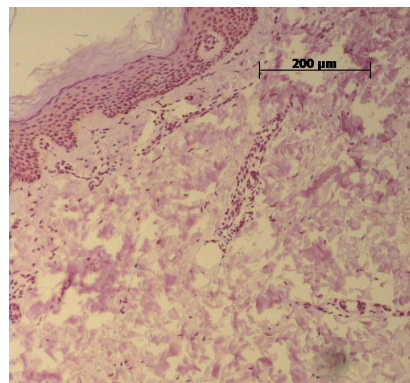
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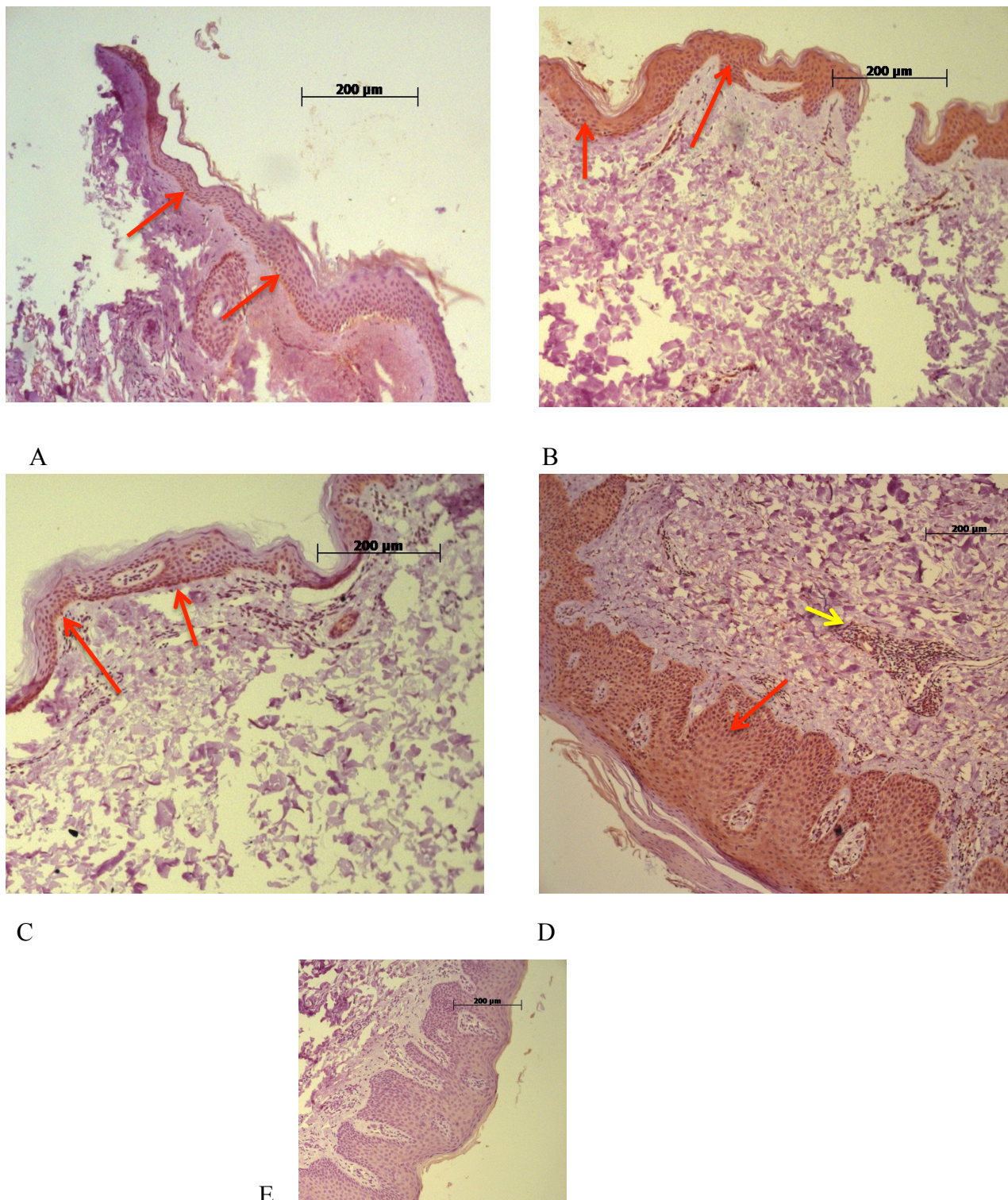


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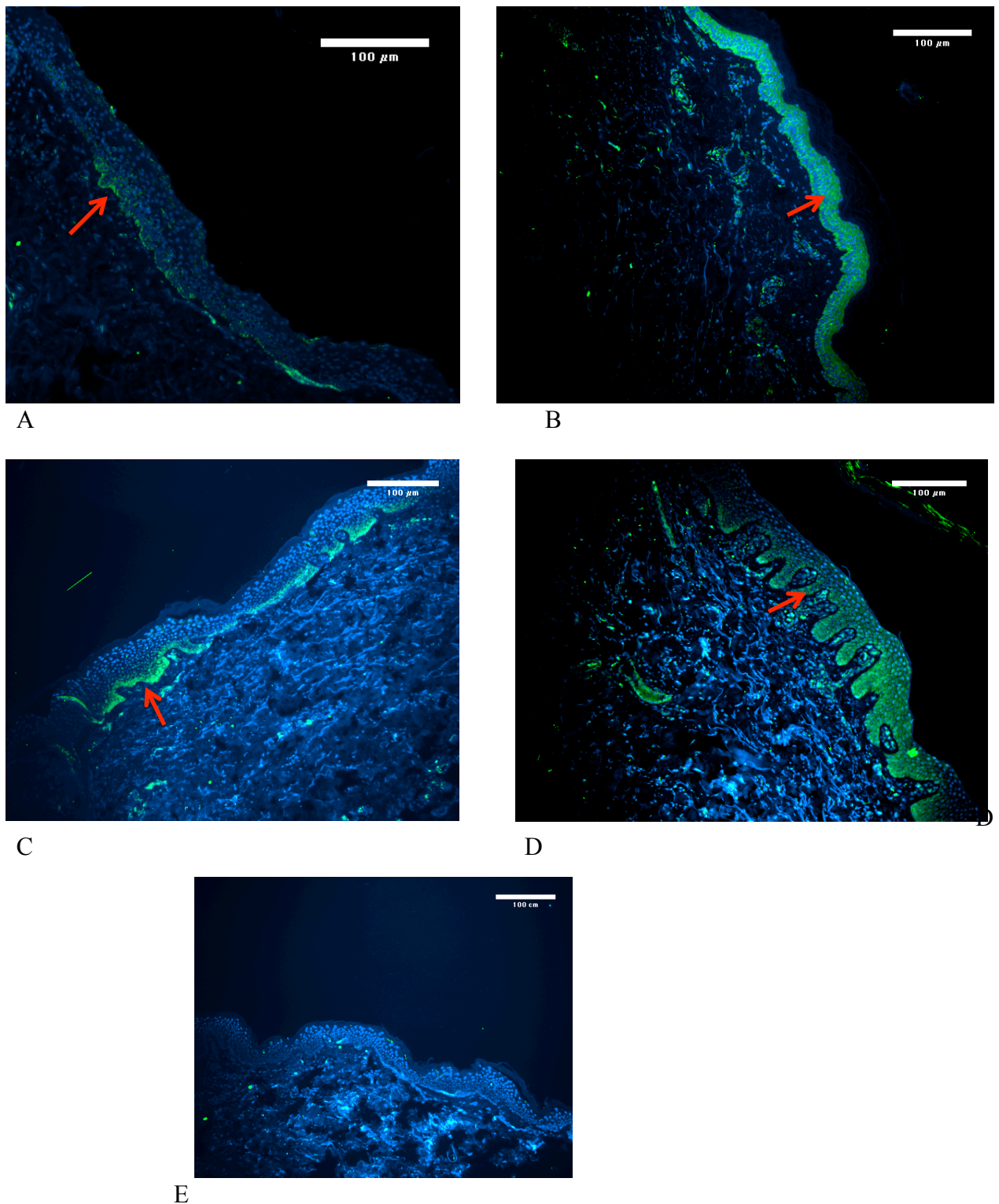
**Figure 3.9. D6 (R&D Systems) monoclonal antibody immuno-staining of healthy control and psoriasis tissue.**

A. Sparse D6 staining on healthy control skin (Red arrows). B. D6 staining found throughout the viable epidermis in uninvolved psoriasis skin (Red arrows). C. D6 staining is restricted to the lower levels of the epidermis in peri-lesional psoriatic skin (Red arrows). D. D6 staining found throughout the viable epidermis in lesional psoriasis (Red arrows). Yellow arrow highlights dermal D6 positive, leukocyte-like cells. E. Isotype control (Rat IgG2a). Images are representative of at least 5 different psoriasis sections, or control sections tested. Scale bar 200μm. All sections were counterstained with haematoxylin and eosin (H&E).





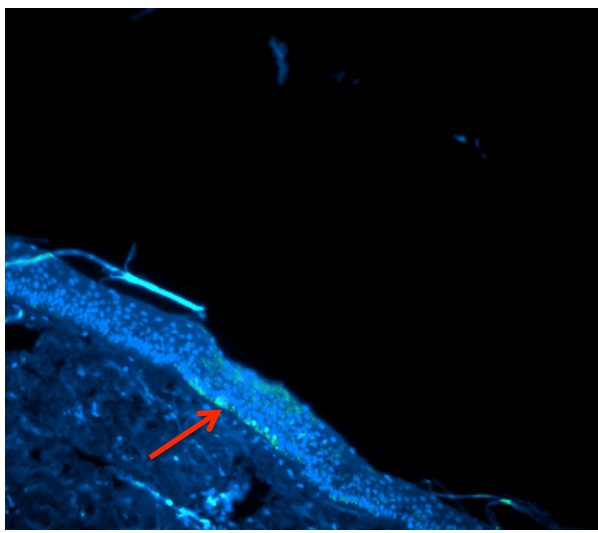
**Figure 3.10. D6 (Sigma) polyclonal antibody immuno-staining of healthy controls and psoriasis tissue.** A. D6 staining is restricted to the lowest levels of the epidermis in healthy controls (Red arrows). B. D6 staining is found throughout the viable epidermis in uninvolved psoriasis sections (Red arrows). C. D6 staining is restricted to the lower levels of the epidermis in peri-lesional skin (Red arrows). D. D6 staining found throughout the viable epidermis in psoriasis lesions (Red arrows). Yellow arrow highlights dermal D6 positive, leukocyte-like cells in psoriasis lesions. E. Rabbit IgG fraction. Images are representative of at least 5 different psoriasis sections, or control sections tested. Scale bar 200µm. All sections were counterstained with haematoxylin and eosin (H&E).



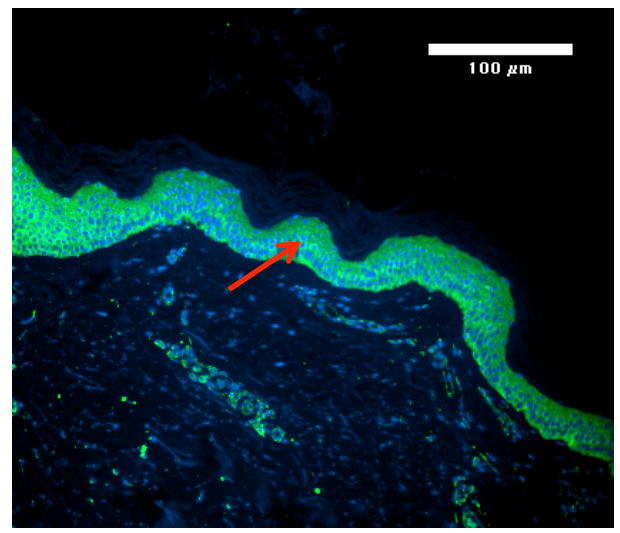
**Figure 3.11. D6 (R&D Systems) monoclonal antibody staining of healthy controls and psoriasis tissue.**

A. Sparse D6 staining on the healthy controls. B. D6 staining is found throughout the viable epidermis in uninvolved psoriasis skin. C. D6 staining is found predominantly towards the lower levels of the epidermis in psoriasis peri-lesional skin. D. D6 staining detected throughout the viable epidermis in psoriasis lesional skin E. Rat IgG2a isotype control (R&D Systems). Nuclei were visualized with DAPI (blue), D6 was visualised with FITC. Images are representative of at least 5 different psoriasis sections, or control sections. Scale bar 100μm.

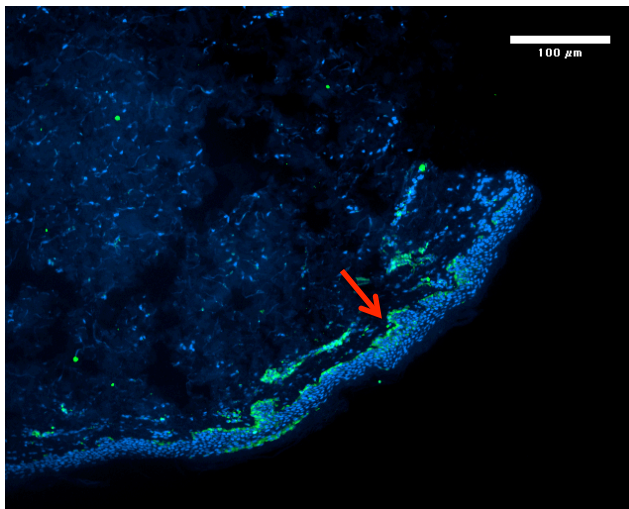




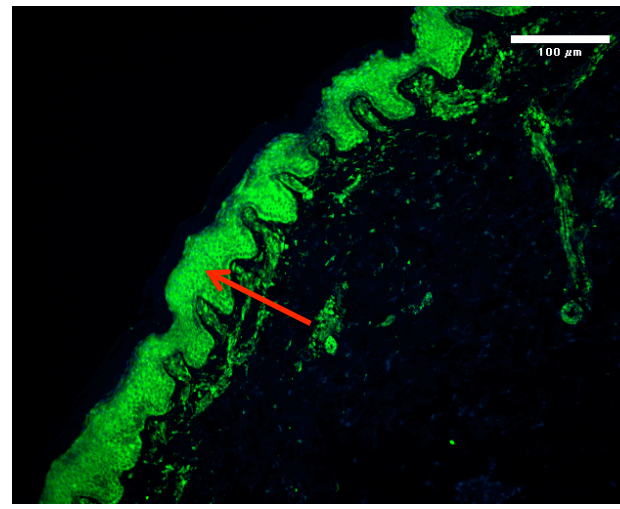
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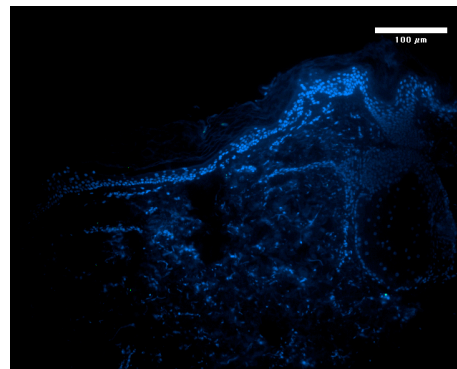
B



C



D



E

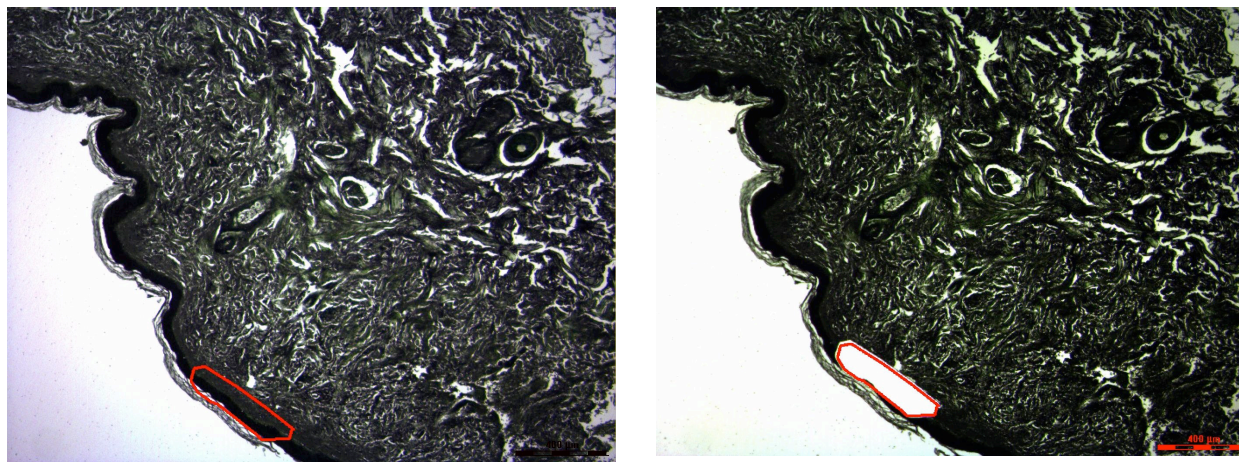
**Figure 3.12. D6 (Sigma) polyclonal antibody staining of healthy control and psoriasis tissue.** A. Sparse D6 staining on the healthy controls. B. D6 staining found throughout the viable epidermis in uninvolved psoriasis epidermis. C. D6 staining is found predominantly towards the lower levels of the epidermis in psoriasis peri-lesional skin. D. D6 staining is found throughout the viable psoriasis lesional epidermis E. Rabbit IgG fraction control (DAKO). Nuclei were visualized using DAPI (blue), D6 was visualized using FITC. Images are representative of at least 5 different psoriasis sections, or control sections. Scale bar 100μm.

Human skin sections incubated with Rat IgG2a (Figure 3.11.E.) or Rabbit IgG fraction (Figure 3.12.E.) had no detectable immuno-reactivity. Therefore, in healthy controls and psoriasis tissue, D6 protein expression is found on the epidermis.

#### 3.4.2. Examination of epidermal D6 expression using laser capture microscopy

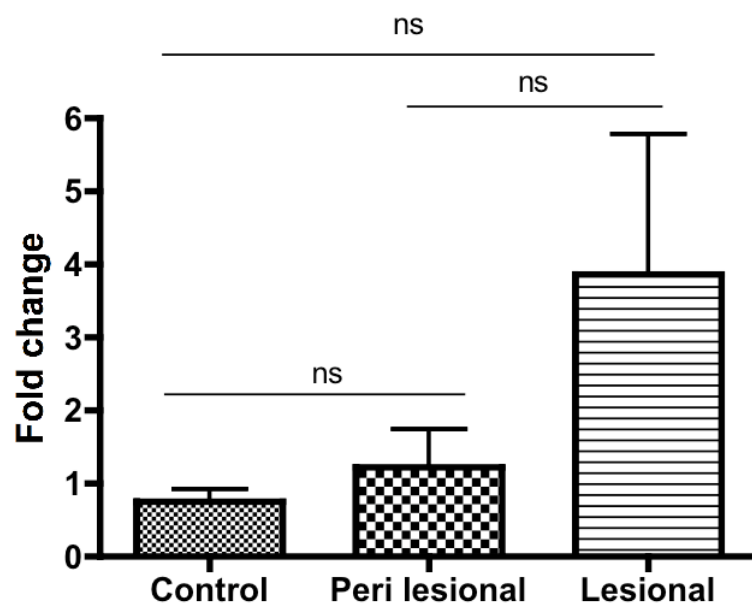
As shown earlier, there is a notable difference in the levels of D6 immuno-reactivity on the epidermal keratinocytes when comparing peri-lesional psoriatic skin (Figures 3.11.C. and 3.12.C.), to lesional psoriatic skin (Figures 3.11.D. and 3.12.D.). Staining in peri-lesional epidermis is largely restricted to the lower levels of the epidermis, whereas D6 staining is found throughout the epidermis in lesional skin. This is at odds with the levels of D6 RNA expression detected in Figure 3.2., as no difference in the levels of D6 RNA expression was reported between peri-lesional, and lesional psoriatic skin. However, Figures 3.9.-3.12. show a marked increase in expression of D6 on the epidermis of peri-lesional and lesional psoriatic skin. To test whether a localised increase in D6 RNA expression occurred specifically in the epidermis of peri-lesional, and lesional psoriatic skin, laser capture microscopy (LCM) was undertaken. This allows for a specific section of tissue to be collected, and then analysis performed on that particular piece of tissue. In Figure 3.13. the tissue section has been cut onto RNase free slides, then stained with Mayer's haematoxylin (Sections were cut onto RNase free slides and stained by Colin Nixon, Beatson Institute). In Figure 3.13.A., the piece of epidermis to be cut using the laser capture microscope is highlighted in red. Figure 3.13.B. shows that the section in red has now been cut from the tissue.

Analysis of D6 transcript levels in control, peri-lesional, and lesional epidermis shows that while there is a notable fold increase in D6 transcript levels comparing healthy control epidermis with lesional epidermis, this did not reach statistical significance. (Figure 3.14.). In addition, there is a general increase in D6 transcript levels comparing peri-lesional epidermis to lesional epidermis (Figure 3.14.), suggestive of a localised increase in D6 expression in the lesional epidermis. Therefore it appears that D6 RNA expression is elevated in the lesional epidermis, although this did not reach statistical significance. However base line D6 RNA expression in the peri-lesional and lesional skin is likely a consequence of expression by an alternative D6 positive cell population.



**Figure 3.13. Image of sections to be dissected using LCM.**

A. Section of the epidermis to be cut is circled in red. B. Section of the epidermis after a section has been cut off. Scale bar 400μm. Sections stained with Mayers Haematoxylin by Colin Nixon, Beatson Institute for Cancer Research.



**Figure 3.14. QPCR analysis of D6 transcript levels in healthy control, peri-lesional and lesional epidermal keratinocytes using LCM.**

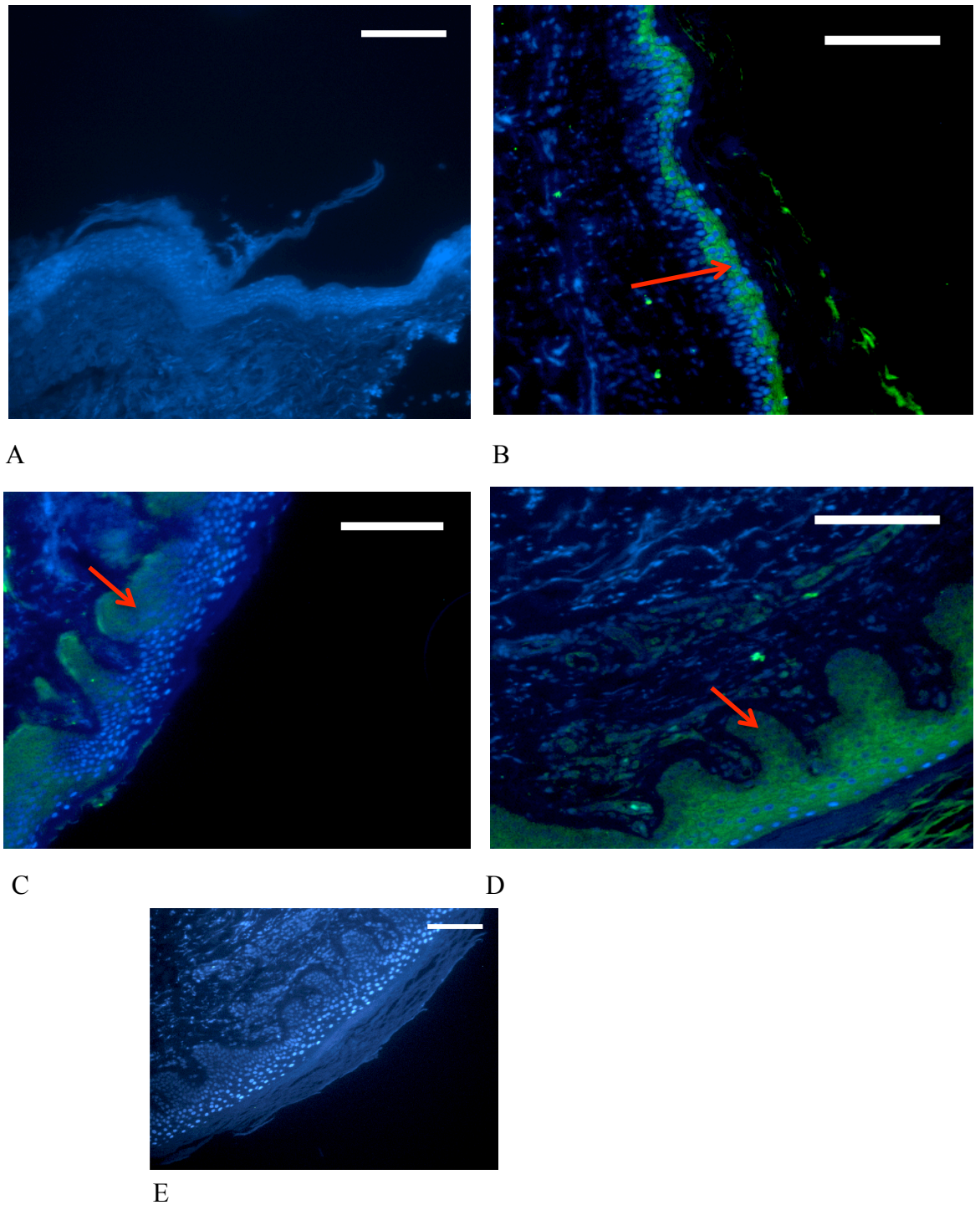
Healthy control epidermis N = 4, Peri-lesional psoriatic epidermis N = 3, Lesional psoriatic epidermis N = 4 Statistical significance was determined using a Kruskal Wallis test, and Dunn's Multiple Comparison Test.

### **3.5. Expression of inflammatory CC chemokines in psoriasis tissue**

A possible reason for the striking elevation of D6 expression in uninvolved psoriatic skin could be to ‘dampen down’, and inhibit, CC chemokine driven inflammation from taking control, and precipitating lesion development. There is a notable loss of D6 expression on the epidermis when comparing uninvolved and peri-lesional psoriatic skin (Figure 3.9 – 3.12). Therefore it is possible that this reduced expression of D6 allows an inflammatory CC chemokine response to take effect, leading to lesion development. If this is the case, it would be expected that uninvolved and peri-lesional psoriatic skin would be immuno-reactive for inflammatory CC chemokines. It is well established that epidermal keratinocytes in psoriasis lesions are immuno-reactive for D6 binding chemokines, including CCL2 and CCL5 (Fukuoka et al., 1998, Raychaudhuri et al., 1999, Giustizieri et al., 2001, Giustizieri et al., 2002). While these studies demonstrated that inflammatory CC chemokines were not produced by keratinocytes in healthy control skin, uninvolved and peri-lesional skin were not examined. To determine whether inflammatory CC chemokines were expressed in uninvolved, and peri-lesional psoriatic skin, immuno-staining for CCL2 and CCL5 was performed.

As can be seen from figures 3.15.A. and 3.16.A., there is a lack of CCL2 or CCL5 expression in healthy controls, confirming previously published results (Fukuoka et al., 1998, Raychaudhuri et al., 1999, Giustizieri et al., 2001, Giustizieri et al., 2002). However, there is detectable staining of CCL2 and CCL5 in uninvolved psoriatic epidermal keratinocytes in Figure 3.15.B. and 3.16.B. (as indicated by red arrows). CCL2 staining as shown in figure 3.15.B. is particularly prominent, localizing to the upper layers of the epidermis. In peri-lesional psoriatic skin, CCL2 and CCL5 immuno-reactivity is detectable in the epidermis, predominantly towards the lower layers of the epidermis (Figure 3.15.C. and Figure 3.16.C.). In the lesional epidermis, there is strong immuno-reactivity for both CCL2 and CCL5 (Figures 3.15.D. and 3.16.D.). Collectively, these data suggest that in uninvolved psoriatic skin, there is on-going, low level inflammation, as evidenced by the detection of inflammatory CC chemokines at this site. Therefore it is possible that D6 expression is elevated in uninvolved skin in an attempt to dampen down CC chemokine driven inflammation, which if unchecked, could result in the inflammatory response taking hold, and the formation of new lesions.

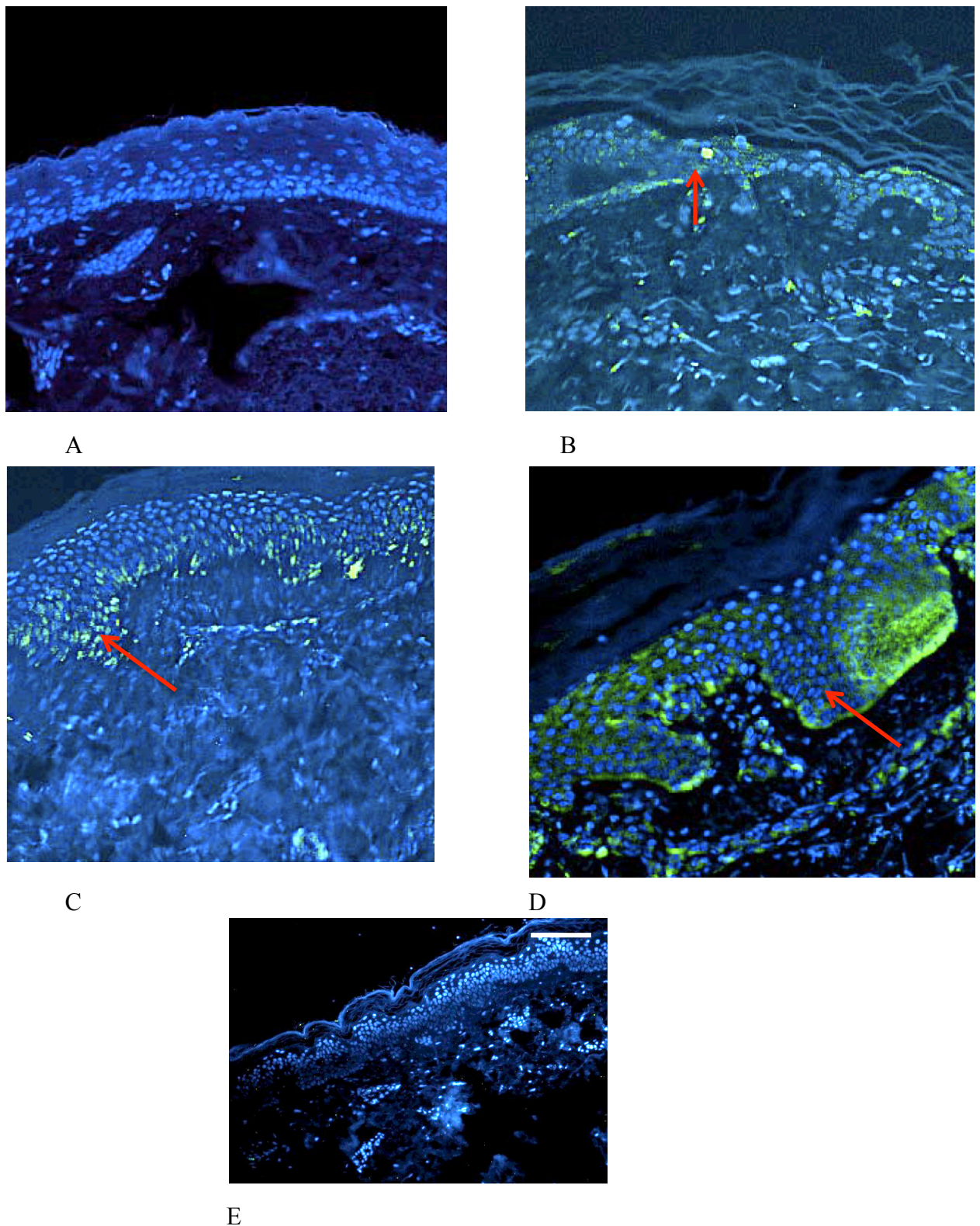




**Figure 3.15. CCL2 immuno-reactivity on the epidermis of healthy control and psoriasis tissue.**

A. No CCL2 immuno-reactivity is detected in healthy control skin. B. CCL2 (FITC) immuno-reactivity is noted on the upper layers of the epidermis (Red arrow) in uninvolved psoriatic skin. C. CCL2 (FITC) immuno-reactivity is found towards the lower levels of the epidermis in peri-lesional skin (Red arrow). D. CCL2 (FITC) immuno-reactivity is found throughout the viable epidermis in psoriasis lesional skin (Red arrow). E. Isotype (Mouse IgG2b) control. Nuclei were visualized using DAPI. Images are representative of at least 4 different sections of each tissue type. Scale bar 100 $\mu$ m.



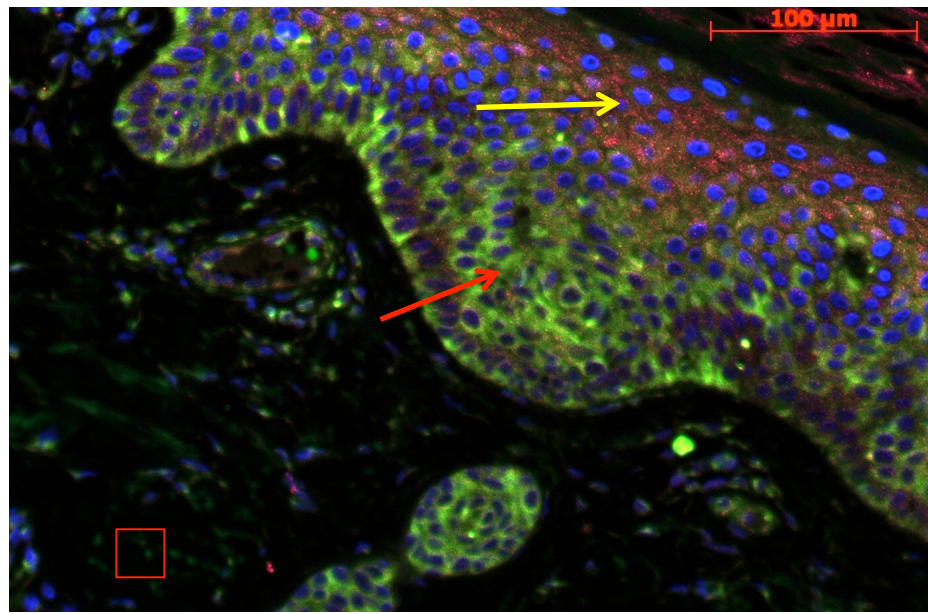


**Figure 3.16. CCL5 immuno-reactivity on the epidermis of healthy control and psoriasis tissue.**

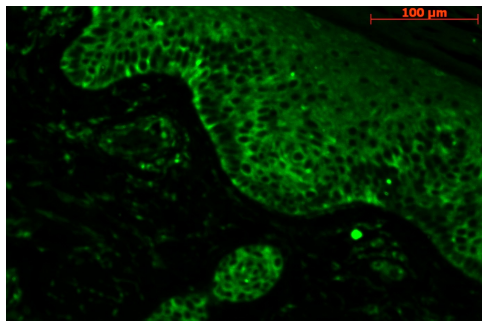
A. No CCL5 immuno-reactivity detected on healthy control skin. B. Weak CCL5 immuno-reactivity (FITC) noted on uninvolved psoriatic epidermis (Red arrow). C. CCL5 immuno-reactivity (FITC) is found towards the lower levels of the epidermis in peri-lesional skin (Red arrow). D. CCL5 immuno-reactivity (FITC) is found throughout the viable epidermis in psoriasis lesional skin (Red arrow). E. Goat IgG fraction control. Nuclei were visualized using DAPI. Images are representative of at least 4 different sections of each tissue type. Scale bar 100 $\mu$ m.

D6 expression has previously been reported on barrier sites, such as the LVs (Nibbs et al., 2001), and the syncytiotrophoblast of the placenta (Madigan et al., 2010, Martinez de la Torre et al., 2007, Nibbs et al., 1997b). Therefore D6 is in position to control the levels of inflammatory CC chemokine release into the tissue. If epidermal D6 expression plays a similar role in controlling inflammatory CC chemokines, it might be expected that D6 would be strongly expressed towards the lower levels of the epidermis. To test this, D6/CCL2 co-staining was performed (Figure 3.17.). In agreement with Figure 3.15.B., CCL2 immuno-reactivity (Cy5) appeared strongest in the upper layers of the epidermis (Figure 3.17.A., see yellow arrow), whereas D6 immuno-reactivity (FITC) was most notable on the lower layers of the epidermis (Figure 3.17.A., see red arrows). This staining pattern fits with the idea of D6 acting as a barrier, limiting the levels of inflammatory CC chemokines entering the dermis from the inflamed epidermis. Both D6 staining (Figure 3.17.B.), and CCL2 staining (Figure 3.17.C.), were found throughout the viable epidermis, confirming data discussed previously (Figures 3.11. – 3.12. for D6 staining, and Figure 3.15. for CCL2 staining).

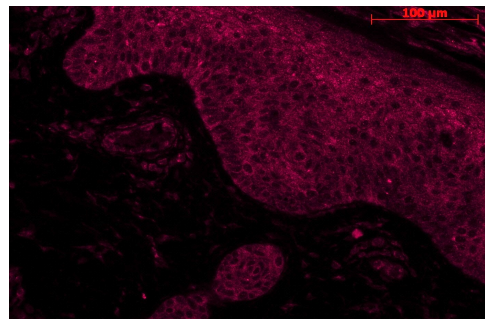
To examine whether D6 and CCL2 expression co-localise to the same part of the section, co-localisation analysis was undertaken. Figure 3.17.D. is a plot of the background fluorescence threshold. The background threshold is set by using a part of the image with minimal immuno-fluorescence (The red box in Figure 3.17.A.). Any fluorescence below these values, is considered to be non-specific fluorescence. The co-stained epidermis is then examined for D6/CCL2 co-expression. Figure 3.17.E. is a plot of D6/CCL2 co-staining in the lesional epidermis. As shown in figure 3.17.E, there are sections of the epidermis that are D6 high, CCL2 low (Yellow box, Figure 3.17.E.), suggestive of strong D6 staining and weak CCL2 staining. In addition, there are sections of the epidermis which are D6 low, CCL2 high (Red box, Figure 3.17.E.), which implies weak expression of D6 and strong expression of CCL2. Use of Pearson's correlation co-efficient, and Mander's overlap co-efficient shows that D6 and CCL2 significantly co-localise to the same part of the tissue, suggesting the sites of D6 and CCL2 expression overlap.



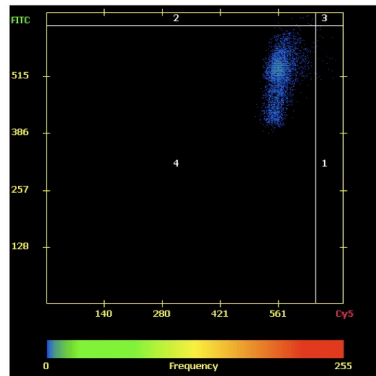
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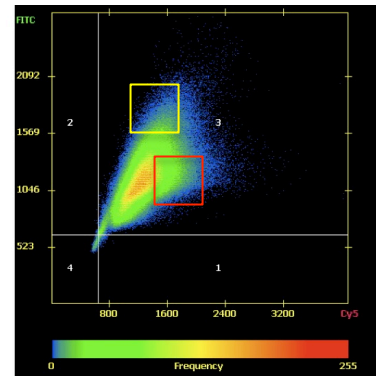
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E

**Figure 3.17. D6/CCL2 co-staining of psoriasis lesional epidermis.**

A. Co-staining of D6/CCL2 with strong D6 (FITC) immuno-reactivity noted towards the lower levels of the epidermis (Red arrow), and strong CCL2 (Cy5) immuno-reactivity towards the upper layers of the epidermis (yellow arrow). B. D6 (FITC) staining of psoriasis lesional epidermis. C. CCL2 (Cy5) staining of psoriasis lesional epidermis. Images are representative of 3 different psoriasis lesions. Scale bar 100μm. D. Fluorescence background, E. D6/CCL2 co-staining. Correlation between D6 and CCL2 was determined using Pearson's correlation co-efficient = 0.612, and Mander's overlap co-efficient = 0.987.

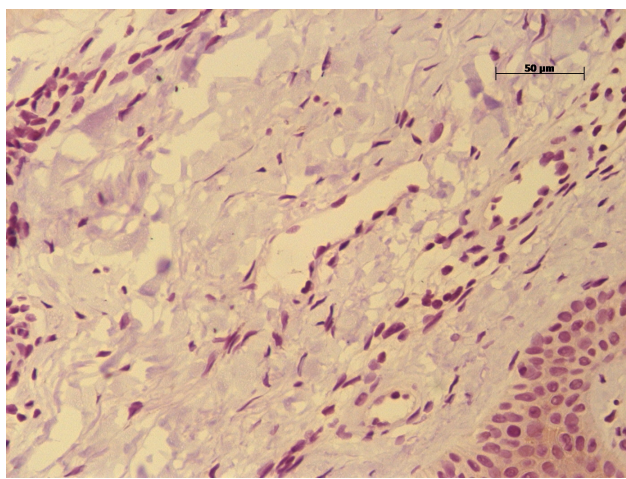
### **3.6. D6 expression on Lymphatic Vessels (LVs) in healthy control and psoriasis**

As shown in section 3.4, the epidermis in uninvolved psoriatic skin, and lesional psoriatic skin, was strongly immuno-reactive for D6. However, Figure 3.2. showed that there was significantly higher levels of D6 expression in uninvolved skin, compared to lesional skin. Suggesting that another site of D6 expression must be partly responsible for the differences observed in the levels of D6 transcripts between uninvolved psoriatic skin and lesional psoriatic skin. As LVs are an established site of D6 expression in the skin, (Nibbs et al., 2001), D6 expression on these cells was analysed next.

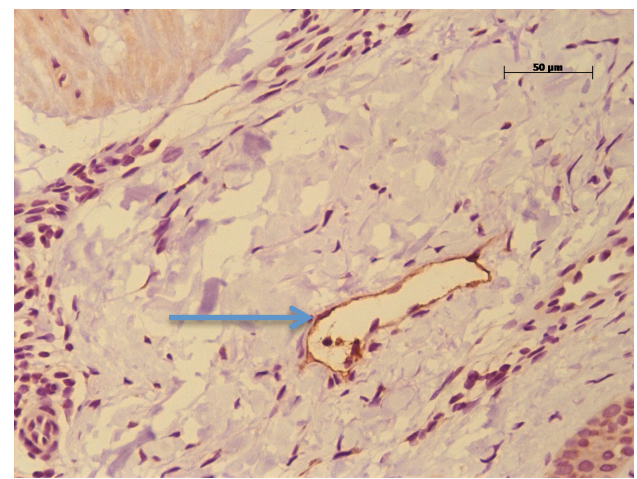
#### **3.6.1. Expression of D6 on LVs in control and psoriasis tissue using D6 R&D Systems antibody**

In order to quantify LV expression of D6, the monoclonal (R&D systems) D6 antibody was used in conjunction with an antibody for the LV marker Podoplanin (Breiteneder-Geleff et al., 1999). Skin sections incubated with rabbit IgG fractions were found to have no background staining (Figure 3.18.A. and C.). As shown in Figure 3.18.B. and D., the antibody for Podoplanin was found to stain specifically for dermal LVs using DAB staining. To co-stain with D6, the detection system was changed from DAB, to IF. No background staining was detected in sections incubated with rabbit IgG fraction (Figure 3.18.E.). The Podoplanin antibody could be used to detect LVs using IF (Figure 3.18.F.). In order to examine D6 expression specifically on the LVs in healthy control sections and psoriasis sections, a co-stain was developed using the D6 monoclonal (R&D systems) antibody, and the Podoplanin (Sigma) polyclonal antibody. An example of a D6 positive, podoplanin co-positive LV is shown in figure 3.19.A. Figure 3.19.B. displays D6 (FITC) positive LV staining, and Figure 3.19.C. displays Podoplanin (Texas Red) positive LV staining. It appears that D6 staining is generally confined to the luminal side of the LVs (highlighted by blue arrows), whereas Podoplanin staining was observed more on the sub-luminal edges of the LVs (highlighted by red arrows). However, D6 expression was also detected on the sub-luminal edges of the LVs (albeit very weak D6 expression), as shown by the 'orange-hue', which indicates co-staining (Figure 3.19.A.)

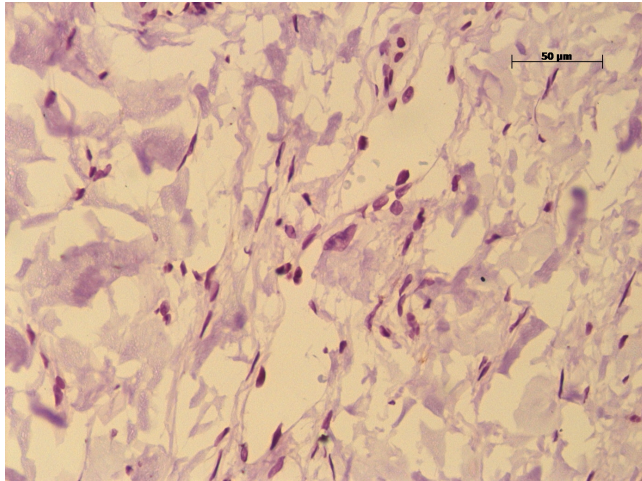




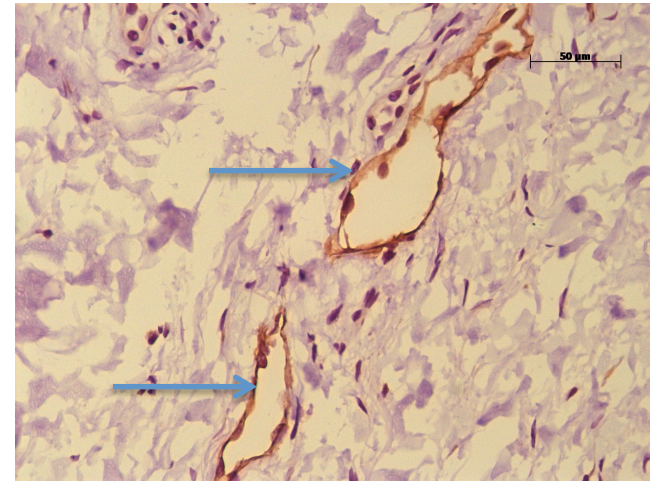
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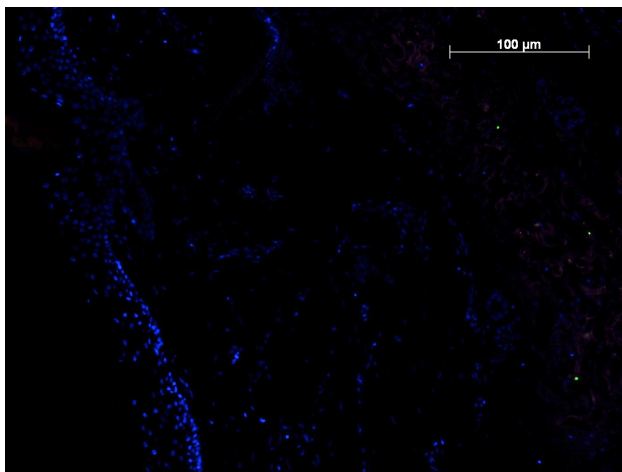
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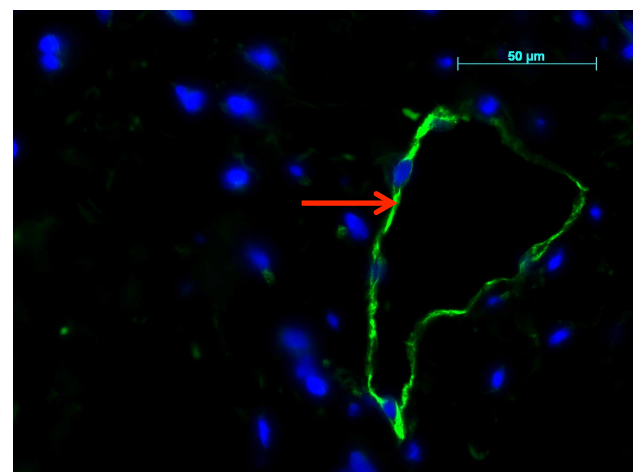
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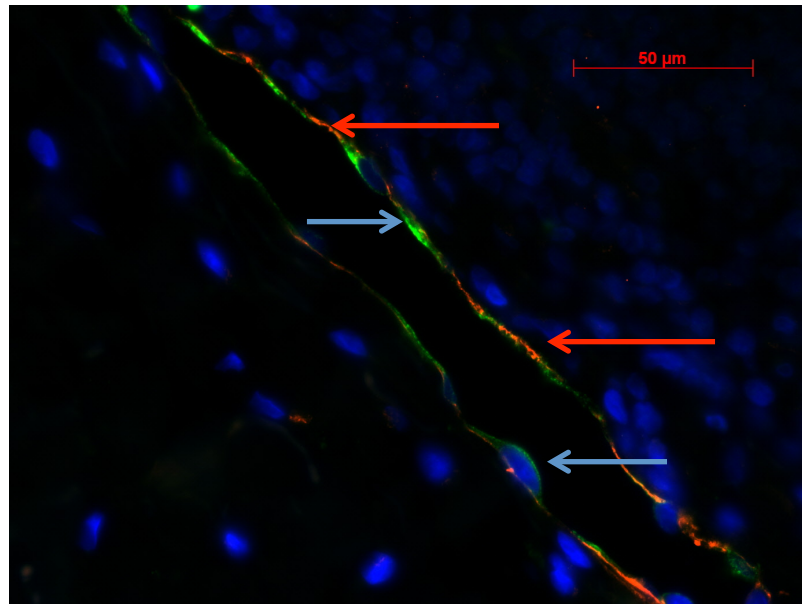
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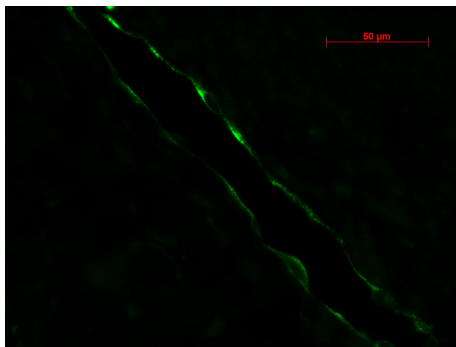
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**Figure 3.18. Podoplanin-positive dermal LVs in human skin.**

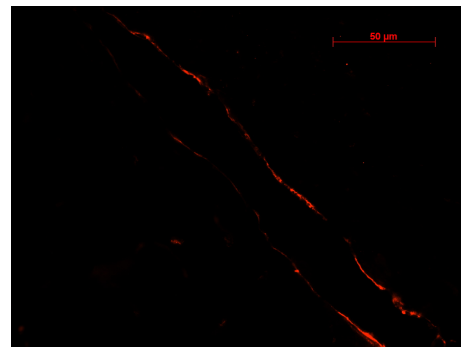
A. Rabbit IgG fraction control. B. Podoplanin immuno-reactivity on LVs (Blue arrow). C. Rabbit IgG fraction control. D. Podoplanin immuno-reactivity on LVs (Blue arrow). E. Rabbit IgG fraction control. F. Podoplanin immuno-reactivity (FITC) on LVs (Red arrow). Scale bars A-D. 50µm. Scale bar E. 100µm, F.50µm



A



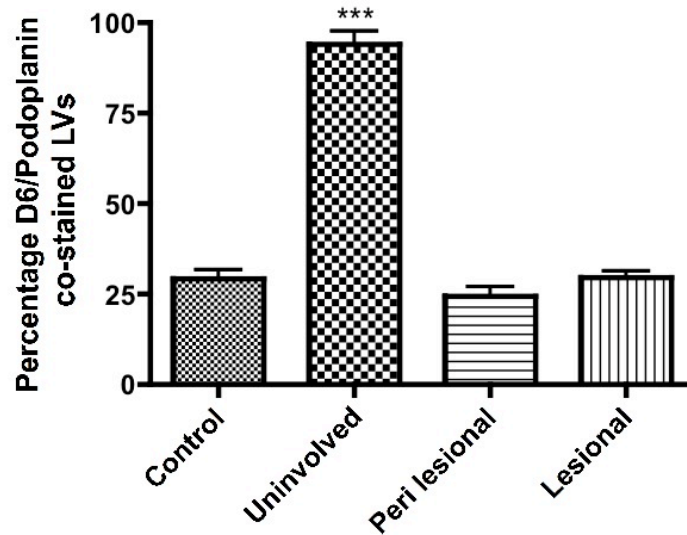
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**Figure 3.19. D6/Podoplanin co-positive dermal LVs in human skin.**

A. The blue arrows point towards D6 (FITC) staining that appears to be biased towards the luminal side of the LV. The red arrows point towards Podoplanin (Texas Red) staining that appears to be biased towards the outer edges of the LV. Scale bar 50μm. B. D6 (FITC) stained LV. Scale bar 50μm. C. Podoplanin (Texas Red) stained LV. Scale bar 50μm.



**Figure 3.20. Percentage of D6/Podoplanin co-positive dermal LVs in healthy controls and psoriasis tissue.**

Control N = 5, Uninvolved N = 5, Peri-lesional N = 5, Lesional N = 5. Kruskal Wallis test and Dunn's Multiple Comparison Test was used to determine significance. Asterisk (\*\*\*) denotes that the percentage of D6/Podoplanin co-stained LVs is significantly higher in uninvolved psoriatic skin compared to healthy control, or peri-lesional psoriatic skin, or lesional psoriatic skin ( $P < 0.0001$ ).

D6/Podoplanin co-staining was then used to stain the various psoriasis tissues and healthy controls, and the expression of LV-D6 quantified. Specifically, all LVs, as determined by podoplanin immuno-reactivity, were counted by examining the entire tissue section. Similarly, the total numbers of D6 positive LVs were counted on the entire tissue section. Finally, the percentage of D6-positive LVs were calculated by dividing the number of D6-positive LVs, by the number of podoplanin-positive LVs, and multiplying this value by 100. These data were then displayed as a percentage of the total LVs (Figure 3.20.). As shown in figure 3.20., a significant increase in the percentage of D6 positive LVs was noted in uninvolved psoriatic skin compared to healthy control skin. Interestingly, there was a significant decrease in the percentage of D6-positive LVs comparing uninvolved skin to peri-lesional skin, and uninvolved skin to lesional skin (Figure 3.20.). Therefore, LV D6 expression, similar D6 RNA expression, is significantly higher in uninvolved skin, compared to control, or peri-lesional and lesional skin. From these data, it is possible that LV-D6 contributes significantly to the elevated numbers of D6 transcripts detected in uninvolved psoriatic skin (Figure 3.2.).

#### 3.6.2. Expression of D6 on LVs in control and psoriasis tissue using D6 Sigma antibody

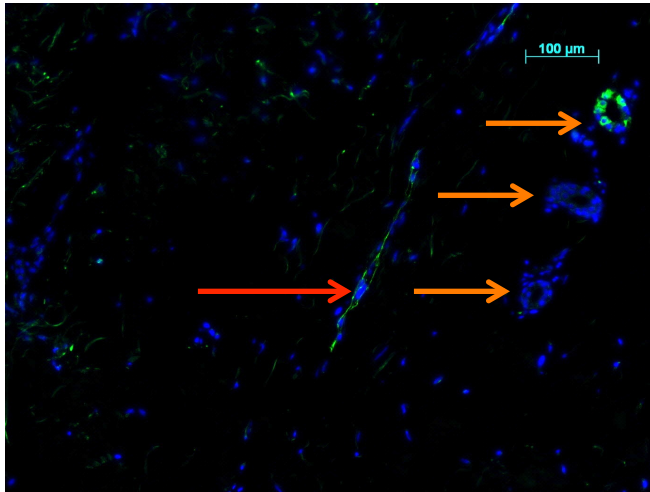
To confirm the data discussed in section 3.6.1., the D6 Sigma antibody was used in conjunction with the Podoplanin antibody to stain serial sections of control and psoriasis tissue. Co-staining was not attempted, as the D6 antibody from Sigma, and the Podoplanin antibody were both polyclonal and raised in rabbit, which would cause problems with secondary detection. Serial sections were therefore stained for either D6 using the polyclonal (Sigma) antibody, or Podoplanin. LVs that were found to stain positive for Podoplanin and D6 were initially imaged at low magnification as shown in Figure 3.21.A. and B. respectively, then confirmed at higher magnification (Figure 3.21.C. and D. respectively). Podoplanin-positive LVs are denoted by a red arrow (Figure 3.21.A. and C.) and D6-positive LVs are marked with yellow arrows (Figure 3.21.B. and D.).

Key to using serial sections stained for different antigens, localizing to a particular structure *in situ*, is the ability to define the surrounding dermis. This allows an accurate analysis of multiple antigens at one specific location in the tissue. The orange arrows in Figure 3.21.A. and B. point to what look like hair follicles in a very similar arrangement. D6 immuno-reactivity on the hair follicles suggests that D6 may be expressed at this site under certain conditions. However, this would require careful validation (similar to the steps taken to validate epidermal D6 immuno-reactivity), and there was not time to do so

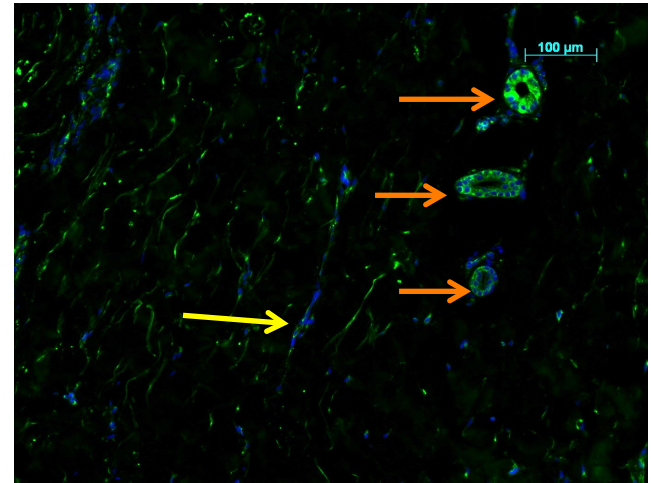


during this project. Thus, using structures in the dermis enables accurate serial sectioning to be performed.

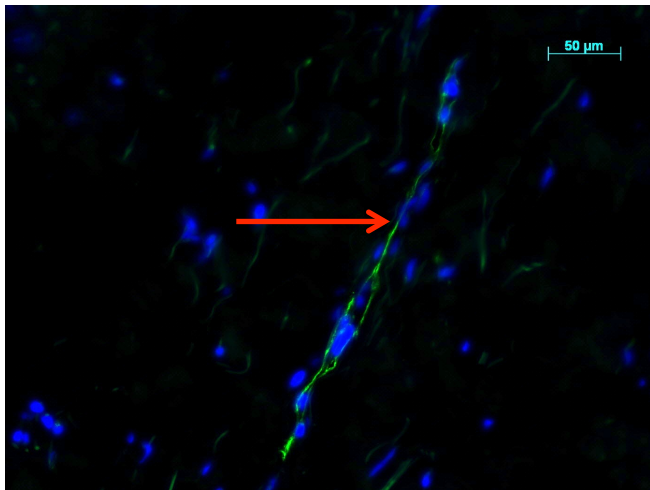
After staining serial sections of healthy controls and psoriasis tissue was complete, the numbers of D6-positive LVs were calculated. To do this, all LVs (as defined by Podoplanin immuno-reactivity) were counted, and then D6-positive LVs counted. The D6-positive LVs were then expressed as a percentage of the total LVs. The data are shown in Figure 3.22. These data confirm the data shown in Figure 3.20. Specifically, a significant increase in the percentage of D6-positive LVs is noted in uninvolved psoriatic skin compared with healthy control skin (denoted in Figure 3.22. by \*). Secondly, there is a significant reduction when the percentage of D6-positive LVs are compared between uninvolved psoriatic skin and peri-lesional psoriatic skin, and uninvolved psoriatic skin and lesional psoriatic skin (denoted in Figure 3.22. by \*\*). Collectively, the data in Figures 3.20. and 3.22. show that in uninvolved psoriatic skin, there is a significantly higher percentage of D6 positive LVs compared to healthy control, peri-lesional or lesional psoriatic skin.



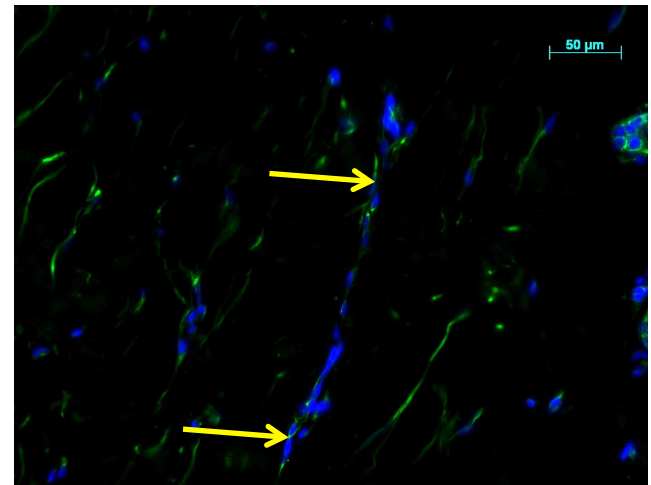
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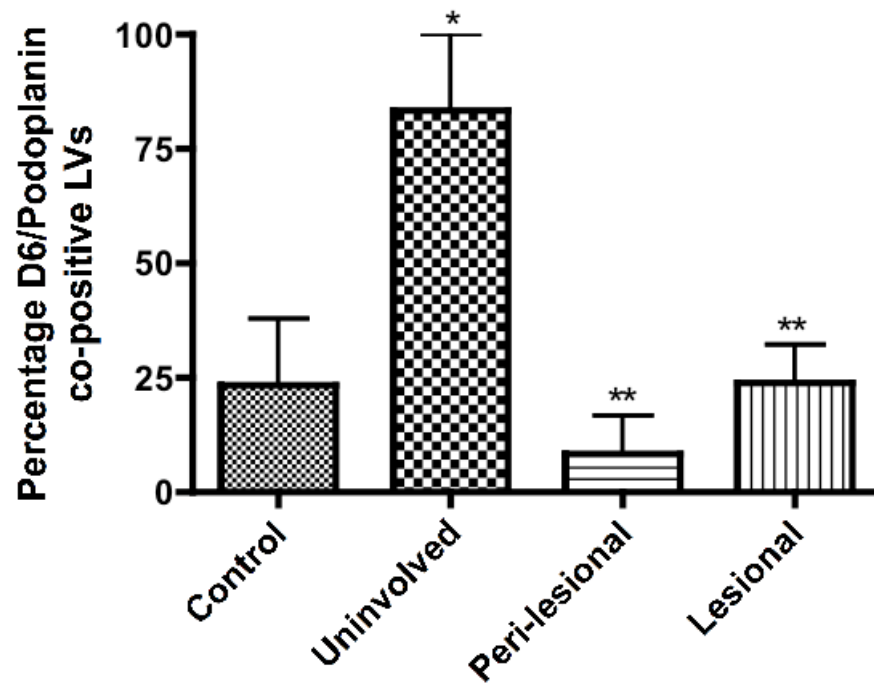
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D

**Figure 3.21. Serial section staining of human skin examining Podoplanin, or D6 polyclonal immuno-staining on LVs.**

A. Podoplanin-positive LV (as denoted by red arrow) or B. D6-positive LV (as denoted by yellow arrow). Hair follicle-like structures found in both serial sections are denoted by orange arrows in A. and B. Scale bar A and B 100μm C. Podoplanin-positive LV (as denoted by red arrow). D. D6-positive LV (as denoted by yellow arrow). Scale bar C and D 50μm.



**Figure 3.22. Percentage of D6 positive, Podoplanin positive LVs in serial stained sections.**

Healthy control or psoriasis tissues were stained with either D6 (Sigma) polyclonal antibody, or Podoplanin antibody. Control N = 4, Uninvolved N = 4, peri-lesional N = 4 and lesional N = 4. \* denotes a significant increase in the percentage of D6 positive, Podoplanin co-positive LVs ( $P < 0.05$ ) in uninvolved skin compared to healthy controls. \*\* denotes a significant decrease in the percentage of D6 positive, Podoplanin co-positive LVs in uninvolved psoriatic skin compared to peri-lesional psoriatic skin, and uninvolved psoriatic skin compared to lesional psoriatic skin ( $P < 0.01$ ). Significance was determined using Kruskal Wallis test and Dunn's Multiple Comparison Test.

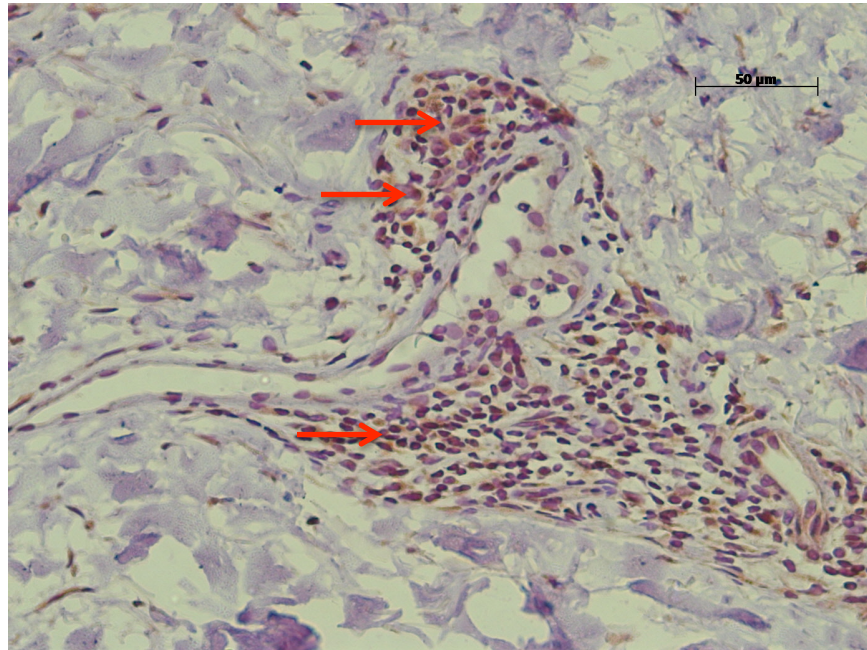
### **3.7. D6 expression on leukocytes in psoriasis**

#### **3.7.1. Identification of D6 positive, leukocyte-like cells**

While investigating D6 immuno-reactivity in the skins of healthy control skin, and psoriasis skin, it was noted that discrete populations of cells, that were not part of the epidermis, and were morphologically distinct from LVs, also stained D6 positive, as shown in Figure 3.23. There appeared to be different numbers of D6 positive cells in the dermis of the various psoriasis tissues and healthy controls. To quantify these D6-positive cells, healthy control skin, and psoriasis skin, were stained for D6 immuno-reactivity using the D6 (Sigma) polyclonal antibody. The total numbers of D6 positive cells were counted by examining the entire immuno-stained section for D6-positive cells, and the results are shown in Figure 3.24. There is a gradual increase in the number of D6-positive cells when healthy controls are compared with uninvolved, peri-lesional, and lesional, psoriatic skin (Figure 3.24.). Significantly more D6-positive cells were noted in psoriasis lesions compared to healthy control (Figure 3.24.). In addition, significantly more D6 positive cells were detected in lesional psoriatic skin compared to uninvolved psoriatic skin. Therefore, the closer to the lesion, the greater the number of D6 positive, leukocyte-like cells that can be detected.

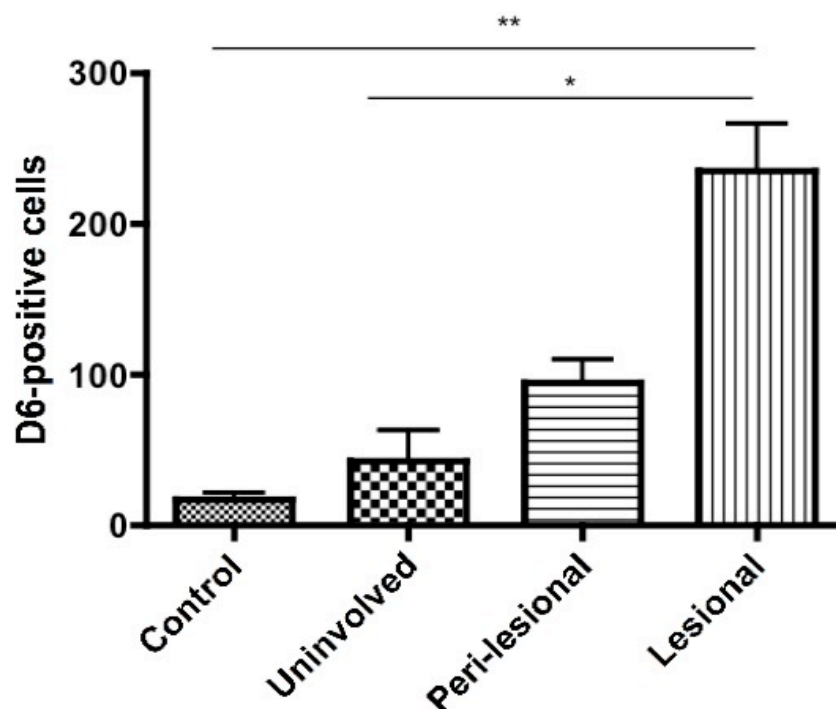
#### **3.7.2. Phenotyping of D6 positive, leukocyte-like cells**

The D6 positive cells in lesional skin were then further characterized. To examine whether D6-positive cells in the lesion were indeed leukocytes, a D6/CD45 co-stain was performed (Figure 3.25.A.). Figure 3.25.B. displays D6 positive cells (FITC), and Figure 3.25.C. displays CD45 positive cells (Texas Red). The majority of D6-positive cells were found to be CD45 co-positive, and therefore can be classified as D6-positive leukocytes (Figure 3.25.A.). In addition, the leukocytes appear to be surrounding a D6 positive LV (see Figure 3.25.A. and B., highlighted with a yellow arrow). The populations of D6 positive leukocytes were then analysed. As pDCs have been shown to have a prominent role in the initiation of psoriasis (Nestle et al., 2005), and D6 expression has also been reported on pDCs (McKimmie et al., 2008), antibody staining was used to confirm their presence in psoriasis lesions. BDCA-2 has been shown to be a marker for pDCs (Dzionek et al., 2001). While no immuno-reactivity was detected in control Goat IgG stained fractions (Figure 3.26.A.), the BDCA-2 antibody was shown to be immuno-reactive against a discrete population of cells (Figure 3.26.B.). To further phenotype D6-positive leukocytes in psoriasis lesions, they were co-stained using the D6 (Sigma) polyclonal antibody, and lineage specific markers (Figure 3.27.). D6 positive cells co-stained with a lineage specific marker.



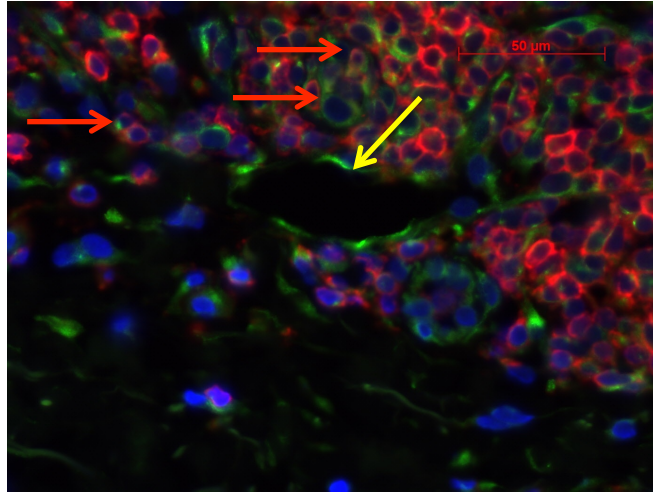
**Figure 3.23. D6 positive cells in the dermis of psoriasis tissue.**

D6-positive cells (stained using D6 Sigma polyclonal antibody) highlighted by red arrows. Scale bar 50μm. Image representative of 8 psoriasis lesional sections tested.

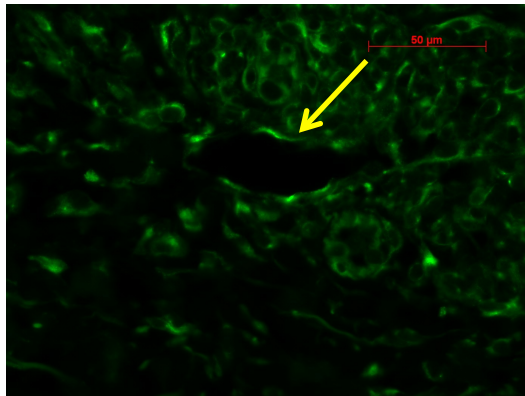


**Figure 3.24. Number of D6-positive dermal cells in healthy controls and psoriasis tissue.** Healthy control N = 4, Uninvolved N = 3, Peri-lesional N = 8, Lesional N = 8. Statistical significance was determined using a Kruskal Wallis test and Dunn's Multiple Comparison Test. \* P<0.05, \*\* P<0.01.

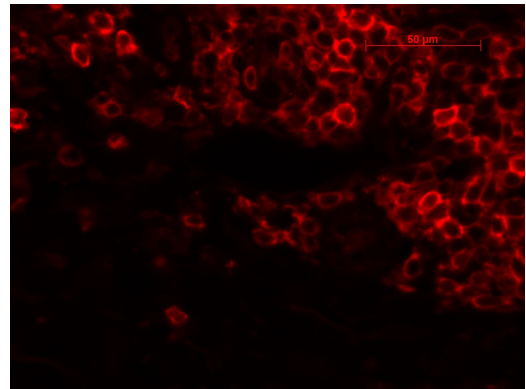




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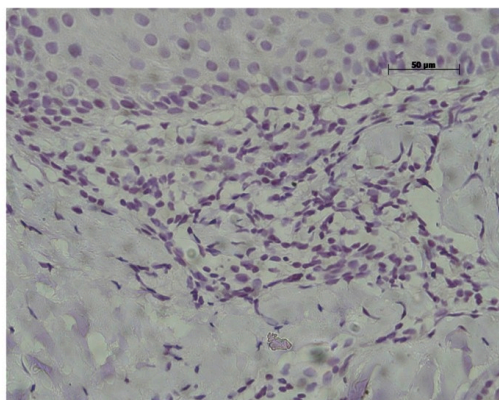
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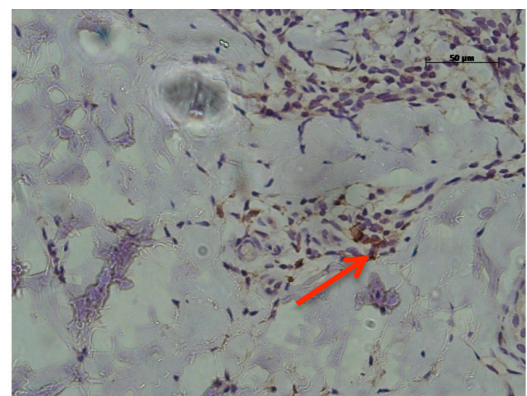
C

**Figure 3.25. D6/CD45 co-positive leukocytes.**

D6/CD45 co-stained cells are indicated by red arrows. A.D6 (FITC)/CD45 (Texas Red) co-stained leukocytes in dermal psoriasis lesions. Co-stained cells highlighted with red arrows. A D6 positive LV is highlighted with a yellow arrow. B. D6 (FITC) immuno-stain C. CD45 (Texas Red) immuno-stain. Scale bars 50μm. Images are representative of 5 different psoriasis lesional sections tested.



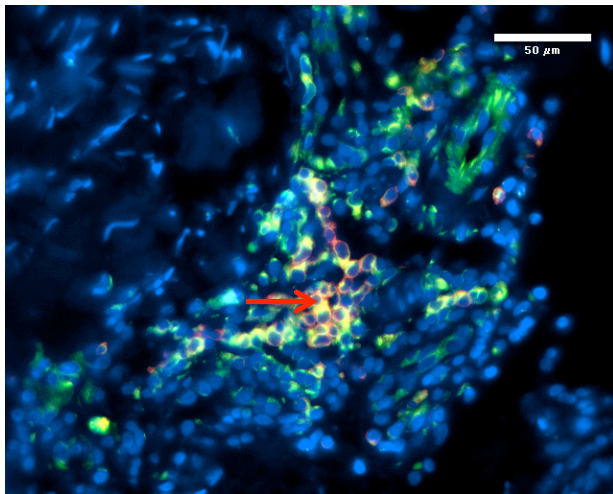
A



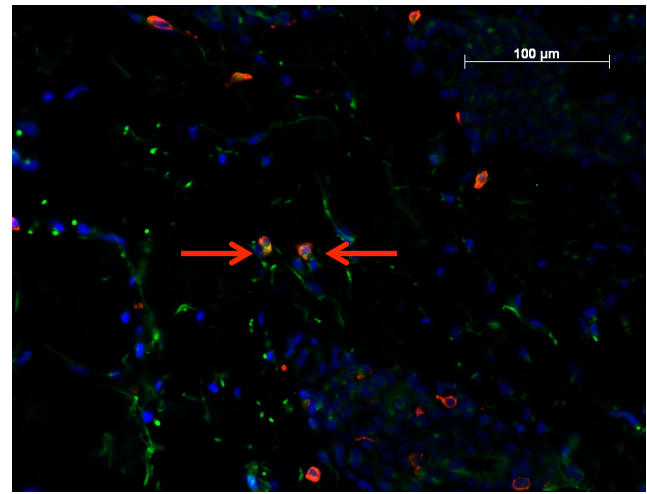
B

**Figure 3.26. BDCA-2 positive cells.**

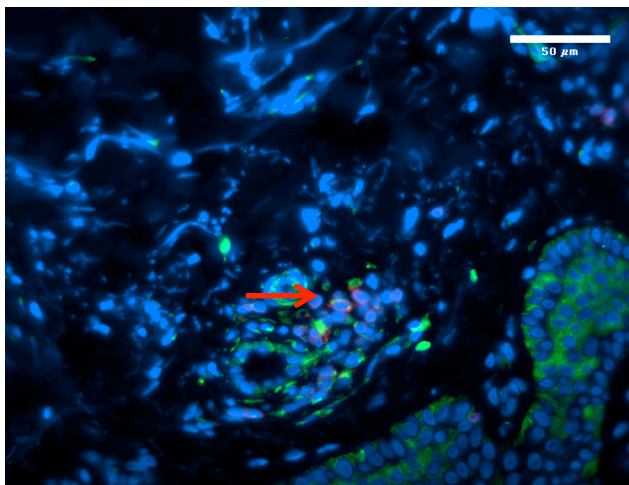
A. Goat IgG fraction or B. BDCA-2 stained cells (Red arrow). The images shown are representative of 4 different psoriasis lesional sections. Scale bar 50μm.



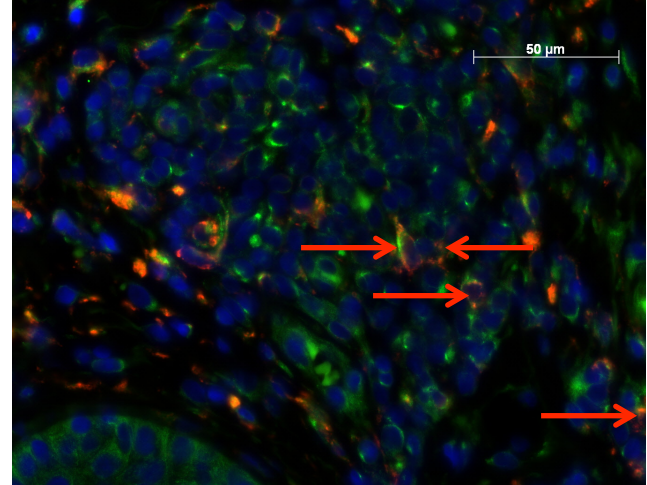
A



B



C



D

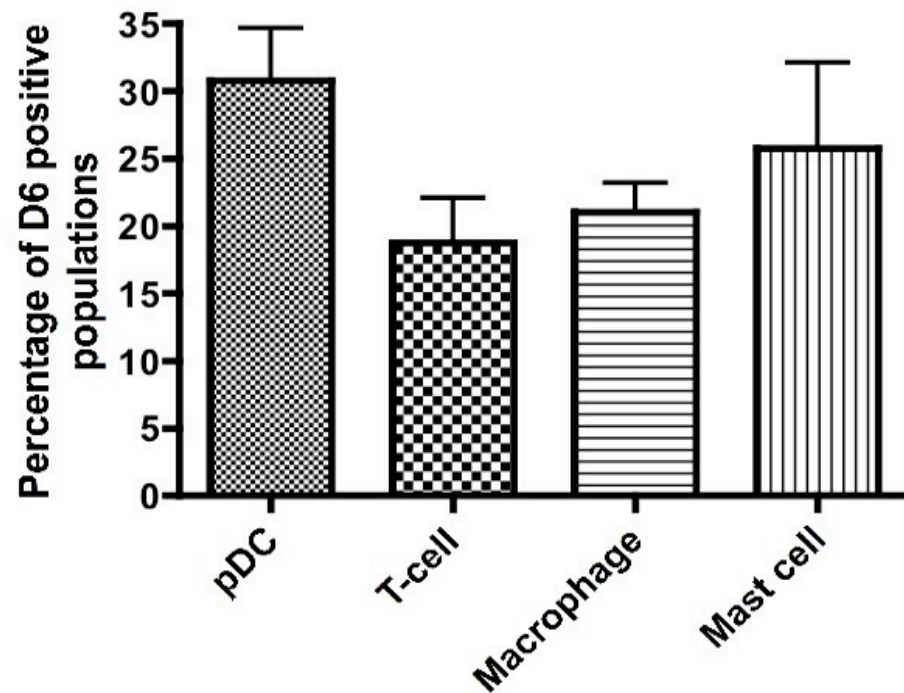
**Figure 3.27. D6 positive leukocytes in psoriasis lesions.**

Leukocytes were stained with D6 (Sigma) polyclonal antibody (FITC) and A. BDCA-2 (pDC marker), B. Tryptase (Mast cell marker), C. CD3 (T-cell marker) or D. CD68 (Macrophage marker). All leukocyte markers were detected using Texas Red as the fluorochrome. Scale bars A, C and D - 50μm Scale bar B - 100μm. D6 co-stained leukocytes are highlighted with red arrows. Images are representative of 4 different psoriasis lesional sections tested.

are highlighted with red arrows. Specifically pDCs with anti-BDCA-2 (Figure 3.27.A), Mast cells with anti-Tryptase (Figure 3.27.B.), T-cells were identified with anti-CD3 antibody (Figure 3.27.C.) and Macrophages with anti-CD68 antibody (Figure 3.27.D.). D6-positive cells were enumerated by examining the dermis of each section, and counting the D6-positive cells.

These leukocyte populations were chosen for analysis as each sub-population of cells has been identified in psoriasis lesions. In order to calculate the percentage of D6-positive cells in each leukocyte sub-population in psoriasis lesions, multiple images were taken of each co-stained section, and the number of D6 positive cells (co-staining with a leukocyte marker, and D6 with no leukocyte co-stain) counted. Then the number of D6/leukocyte double positive cells expressed as a percentage of total D6 positive cells by dividing the number of D6/leukocyte double positive cells, by the total number of D6 positive cells. This number was then multiplied by 100, and the value entered into the graph. These data are shown in figure 3.28. Psoriasis lesions have a variety of D6 positive populations with no one-leukocyte population dominating. The percentages of each D6 positive leukocyte population were added together to calculate the approximate percentage of all D6 positive cells identified. The majority of D6 positive cells were accounted for (>96%) by this staining approach. Thus in psoriasis lesions, D6 is expressed by a variety of leukocyte populations, suggesting that D6 may have a function on leukocytes in the context of disease.





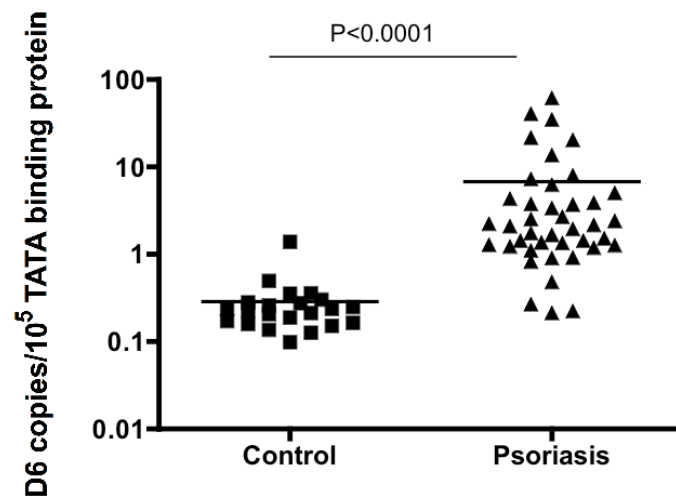
**Figure 3.28. Percentage of each D6-positive leukocyte population in psoriasis lesions.**

The above data were generated by examining multiple fields of view in each stained lesional section, and counting all D6 positive, leukocyte-like cells (positive and negative for leukocyte co-stain). The data were then converted into a percentage, and displayed above. 4 different psoriasis lesional sections were used for each of the above co-stains.

### **3.8. D6 expression on circulating peripheral blood mononuclear cells (PBMCs) in psoriasis**

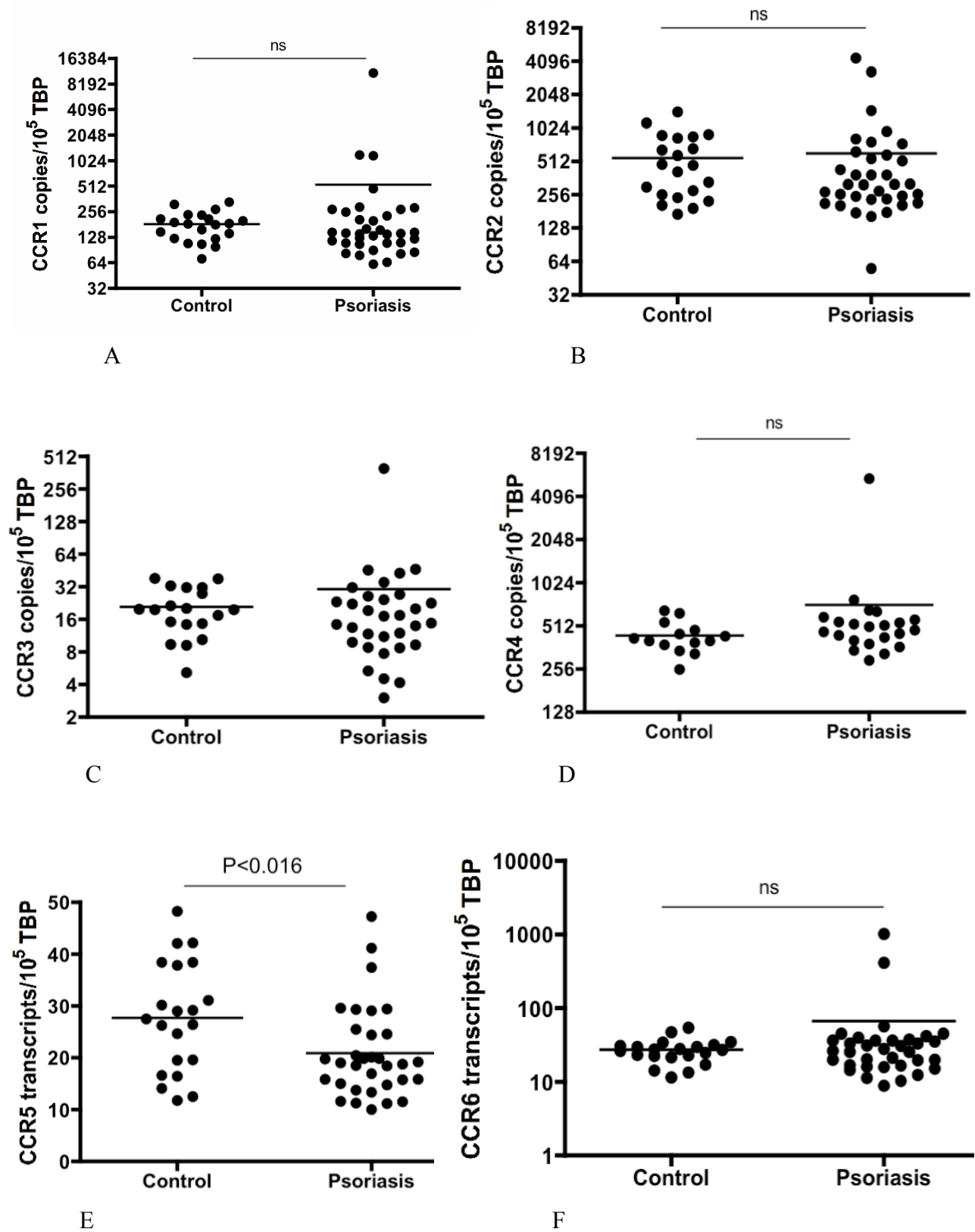
As shown in figure 3.2., there is a significant, and notable increase in the number of D6 transcripts in uninvolved psoriatic skin compared with healthy control, or peri-lesional and lesional psoriatic skin. Therefore, at a site distant from the lesion, D6 is elevated (possibly to inhibit low level inflammation at this site). To test whether elevated D6 expression at a site away from the lesion occurs only in the skin, or if elevated D6 expression is a general response at sites far removed from the lesion, another non-lesional site was examined for D6 expression. As circulating PBMCs are distant from the lesion, these were used as a second non-lesional site of D6 expression. PBMCs from healthy controls and psoriasis patients were obtained, the RNA processed and purified, and D6 transcript numbers measured using QPCR. As shown in figure 3.29., there is an approximately 10 fold, significant increase in the numbers of D6 transcripts in circulating PBMCs from psoriasis patients compared with healthy controls. Therefore, at sites away from the psoriatic lesion, D6 expression is significantly elevated.

To examine whether other chemokine receptors with known roles in inflammation were elevated or reduced in psoriasis PBMCs compared with healthy control PBMCs, QPCR was used to measure the expression levels of CCR1-CCR6, and CXCR1-CXCR3. Figures 3.30. and 3.31. show that D6 appears to be rather unique in terms of a major transcriptional dysregulation in psoriasis PBMCs compared to healthy control PBMCs. Almost all the chemokine receptors analysed showed no difference in expression levels between healthy control PBMCs and psoriasis PBMCs. However, in psoriasis PBMCs, there was a modest, but significant, reduction in the number of CCR5 transcripts compared with healthy control PBMCs (Figure 3.30.). Thus, elevated D6 expression in psoriasis patient PBMCs suggests a prominent role for D6 in the pathogenesis of psoriasis.



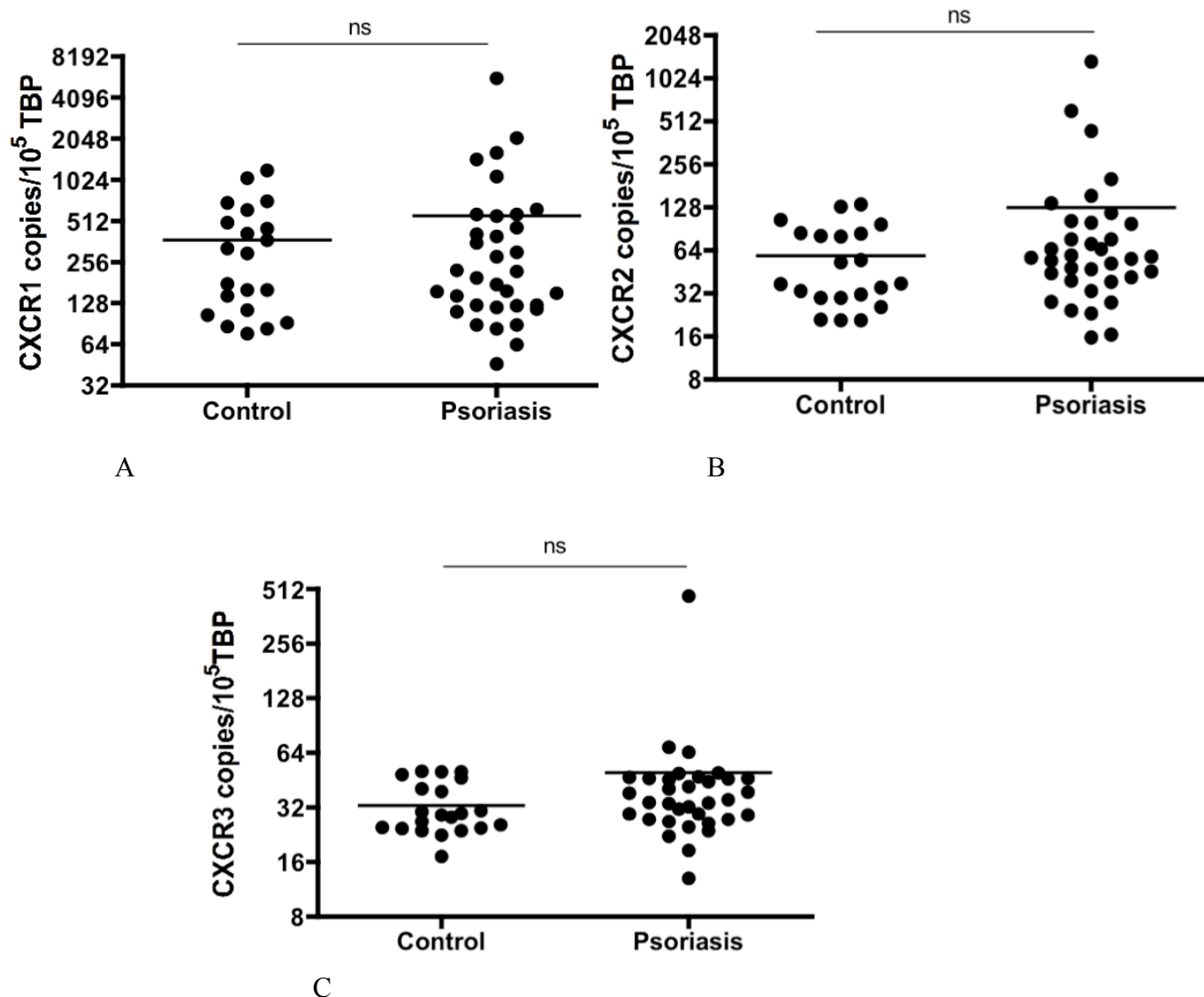
**Figure 3.29. QPCR analysis of the numbers of D6 transcripts in circulating PBMCs of healthy controls and psoriasis patients.**

Healthy control N = 21 Psoriasis N = 38 Statistical significance was determined using a Mann Whitney U test.



**Figure 3.30. QPCR analysis examining the levels of CC chemokine receptor transcripts in healthy control and psoriasis PBMCs.**

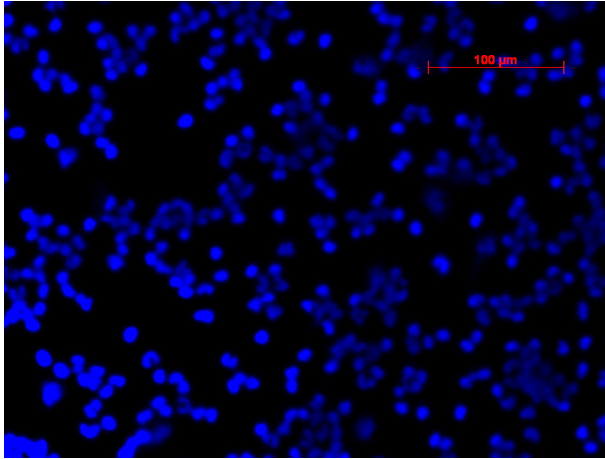
Control N = up to 21 Psoriasis N = up to 35. Statistical significance was determined using a Mann Whitney U test.



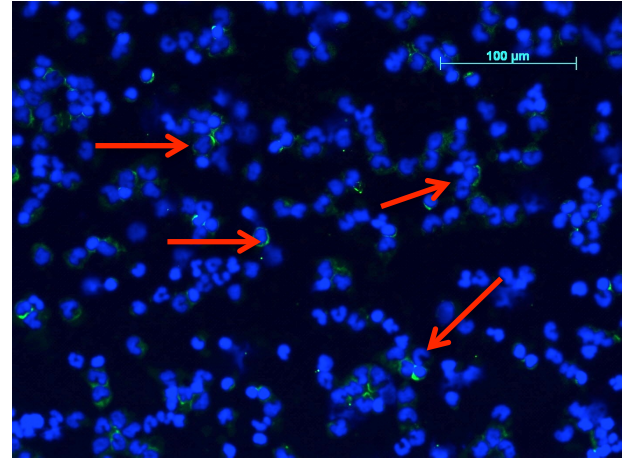
**Figure 3.31. QPCR analysis examining the levels of CXC chemokine receptor transcripts in healthy control and psoriasis PBMCs.**

Control N = 21 Psoriasis N = 35. Statistical analysis was determined using a Mann Whitney U test.

To examine whether an increase in D6 protein occurred in conjunction with elevated D6 transcript numbers in psoriasis PBMCs, an antibody stain for D6 was developed for cytopun PBMCs. PBMCs were isolated from buffy coats using ficoll-mediated separation. These cells are healthy controls as they are taken from volunteers donating blood for transfusions. The cells were then spun onto slides and examined for D6 immuno-reactivity using the D6 (Sigma) polyclonal antibody (Figure 3.32.). No staining was detected in cytopun PBMCs incubated with Rabbit IgG fractions (Figure 3.32.A.). As displayed in Figure 3.32B, D6 immuno-reactivity was detected on PBMCs. With staining for D6 on cytopun leukocytes confirmed, PBMCs from healthy controls (Figure 3.33.A.) and psoriasis patients (Figure 3.33.B.) were examined for D6 protein expression. As shown in Figure 3.33.C., significantly more D6-positive PBMCs were observed in psoriasis circulating patients compared with healthy controls. Therefore, elevated D6 expression at sites distant from the psoriasis lesion is not restricted to uninvolved psoriatic skin, and is suggestive of a general up-regulation of D6 in non-lesional sites, possibly in an attempt to contain the aberrant inflammatory response found in the lesions.



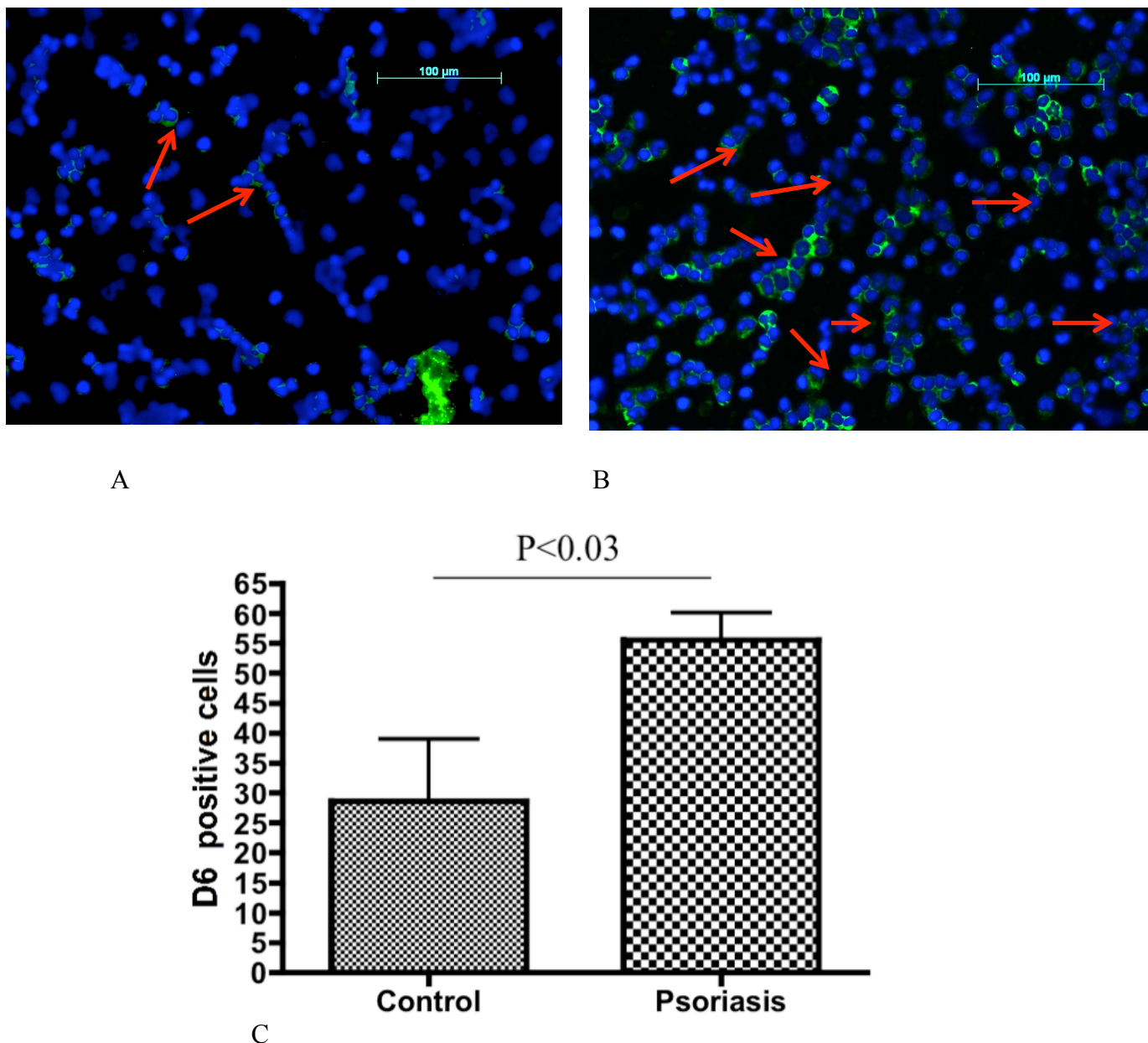
A



B

**Figure 3.32. D6 positive cells in circulating PBMCs.**

A. Rabbit IgG fraction control (DAKO) or B. D6 (Sigma) polyclonal stained PBMCs (highlighted with red arrows). Scale bars 100μm.



**Figure 3.33. D6 positive PBMCs in healthy control or psoriasis patients.**

A. Healthy control D6-positive PBMCs or B. Psoriasis D6-positive PBMCs were analysed for D6 expression using D6 (Sigma) polyclonal antibody. C. Number of D6 positive cells in healthy controls, and psoriasis patients. The cells were enumerated by counting D6-positive cells in 10 different fields of view per stained section, and taking the average value. Healthy control N = 3, Psoriasis N = 10. D6 positive immuno-staining is highlighted using red arrows. Statistical significance was determined using a Mann Whitney U test.



### **3.9. Summary of chapter 3 – D6 in psoriasis**

As discussed in section 1.9. of the introduction, D6 is a chemokine-scavenging receptor, which can bind to, and internalize at least 14 inflammatory CC chemokines *in vitro* (Graham and Locati, 2013). To analyse the function of D6 *in vivo*, D6 KO mice were generated. While the D6 KO mice had no resting phenotype, application of phorbol ester to the shaved dorsal skin of D6 KO mice resulted in severe inflammation, whereas in WT mice only a very transient and mild inflammatory response was noted (Jamieson et al., 2005). In inflamed D6 KO mice, one of the features was delayed clearance of inflammatory CC chemokines (Jamieson et al., 2005), i.e. the very chemokines D6 has been shown to bind to and internalize *in vitro* (Graham and Locati, 2013). From a histological stand point, inflamed D6 KO mouse skin resembled the human disease psoriasis (Jamieson et al., 2005). The data from Graham and colleagues (Jamieson et al., 2005) lead to the hypothesis,

‘A loss of D6 expression in uninvolved psoriatic skin is associated with the development of psoriatic lesions’.

The aim of this chapter was to test this hypothesis. Biopsies were obtained from histologically normal, ‘uninvolved’ psoriatic skin, in addition to an elliptical biopsy which covered peri-lesional psoriatic skin (directly adjacent to the lesion) and lesional psoriatic skin. Control skin was obtained from healthy volunteers. QPCR analysis of D6 expression in healthy control and psoriatic skin showed that D6 expression is significantly elevated in psoriatic skin compared to healthy controls. Strikingly, D6 expression was significantly elevated in uninvolved psoriatic skin compared to healthy control skin, peri-lesional or lesional psoriatic skin. Therefore, the hypothesis was proved to be accurate.

To examine which D6-expressing cells the increase in D6 RNA expression in psoriatic skin localised to, D6 immuno-staining was performed. To ensure consistent results, two separate D6 antibodies were used, and their specificity for D6 confirmed using the LVs and the placenta, both established sites of D6 expression (Martinez de la Torre et al., 2007, Nibbs et al., 2001, McKimmie et al., 2013). Immuno-staining for D6 showed that D6 localised to the epidermal keratinocytes, a previously unidentified site of D6 expression. In healthy control skin, D6 expression was sparse, and localised exclusively to the stratum basale, whereas in uninvolved psoriatic skin, D6 was found throughout the viable epidermis. Therefore, increased D6 RNA expression in uninvolved psoriatic skin occurred concomitantly with an increase in epidermal-D6 protein. In peri-lesional psoriatic tissue, epidermal-D6 protein was again localised to the lowest level of the epidermis. However,

D6 expression on the epidermis in peri-lesional psoriatic skin was more uniform (i.e. found on the majority of the stratum basale) compared to healthy control skin. Therefore, a reduction in D6 RNA expression, and D6-protein expression, occurred when comparing uninvolved psoriatic skin and peri-lesional psoriatic skin.

However, in lesional psoriatic skin, D6 expression is once again found throughout the viable epidermis. Therefore a significant reduction in D6 RNA expression was observed when comparing uninvolved psoriatic skin and lesional psoriatic skin, but D6 protein expression was found throughout the viable epidermis in both uninvolved and lesional psoriatic skin. This apparently contradictory result led us to examine D6 expression in an established site of cutaneous D6 expression, the LVs (Nibbs et al., 2001), as it is possible the LVs could be contributing to the striking increase in D6 RNA levels in uninvolved psoriatic skin. Co-staining for D6 and the lymphatic endothelial cell marker podoplanin (Breiteneder-Geleff et al., 1999) provided two useful insights into D6 on LVs. Firstly, D6 also expression is biased towards the lumen of the LV, although weak D6 expression was detected in the sub-luminal side of the LV. Secondly, enumeration of D6 positive LVs showed there was a significant reduction in the percentage of D6 positive LVs in lesional psoriatic skin, compared to uninvolved psoriatic skin. Therefore in uninvolved psoriatic skin, all of the viable epidermis, and almost all of the LVs were D6 positive. These data suggest that LV-D6 contributes significantly to the increase in D6 RNA levels in uninvolved psoriatic skin.

To examine why D6 expression was so notably elevated in uninvolved psoriatic skin, immuno-stains for CCL2 and CCL5 were performed. While no CCL2 or CCL5 was detected in healthy controls, the epidermis of uninvolved psoriatic skin was immuno-reactive for both CCL2 and CCL5. As uninvolved psoriatic skin has no obvious histological abnormalities, CCL2 and CCL5 at this site are apparently unable to mediate the recruitment of leukocytes to the skin. Therefore, increased D6 expression in uninvolved psoriatic skin may be involved in 'dampening-down' the inflammatory CC chemokine driven immune response. In peri-lesional psoriatic skin, the inflammatory CC chemokine expression appears to be localised towards the lower layers of the epidermis, suggesting that a transient loss of D6 expression may allow release of inflammatory CC chemokines into the dermis. Finally in the epidermis of psoriatic lesions, inflammatory CC chemokines are found throughout the viable layers (similar to D6). Interestingly, co-staining for D6 and CCL2 showed that while both proteins are found throughout the epidermis, the strongest area of D6 expression localizes to the lower layers of the

epidermis, whereas immuno-staining for CCL2 was strongest in the upper layers of the epidermis. These data suggest that D6 on the epidermis forms a barrier, restricting the release of inflammatory CC chemokines into the dermis of the psoriatic lesion.

Interestingly, in healthy control skin and psoriatic skin, particularly lesional psoriatic skin, D6 positive cells were detected in the dermis. These cells were morphologically distinct from LVs. Using co-staining it was subsequently identified that these D6 positive cells were leukocytes. Even though a loss of 'global' D6 expression occurs in full thickness biopsies obtained from lesional psoriatic skin compared to uninvolved psoriatic skin, significantly more D6 positive leukocytes are found in psoriatic lesions compared to uninvolved psoriatic skin. It is possible that leukocyte-D6 expression is responsible for organizing the spatial positioning of leukocytes in the lesion, and this is discussed in more detail in section 6.1.1.4. The increased PBMC-D6 expression in psoriatic patients compared to healthy controls is of interest as it has been detected in SSc (Codullo et al., 2011), and RA (Helen Baldwin, personal communication), which suggests that increased PBMC-D6 expression may be a general marker of inflammation.

To sum up, at sites not directly involved in the pathology of psoriasis, i.e. uninvolved psoriatic skin, and circulating PBMCs, D6 is strikingly elevated.

## **Chapter 4 – Regulation of D6 expression**

## 4. Regulation of D6 expression

### 4.1. Introduction

Since its initial identification in 1997 (Nibbs et al., 1997a, Nibbs et al., 1997b), many insights into D6 biology, and its role(s) within the context of inflammatory disease have been obtained. It is currently established that D6 is a chemokine scavenger, i.e. it can bind to, internalize, and target for degradation, at least 14 inflammatory CC chemokines (Graham, 2009). The prominent sites of D6 expression *in vivo* have also been defined. The placenta is believed to be a major site of D6 expression (Nibbs et al., 1997b), in addition to the LVs (Nibbs et al., 2001). Many leukocyte subsets are now known to be D6-positive (Hansell et al., 2011, McKimmie et al., 2008) and this study has found that epidermal keratinocytes also express D6, particularly within the context of psoriasis (See chapter 3). *In vivo*, it appears that D6 has a role in resolving inflammatory CC chemokine driven inflammation (Jamieson et al., 2005, Martinez de la Torre et al., 2007, Martinez de la Torre et al., 2005, Madigan et al., 2010, Lee et al., 2011, Nibbs et al., 2007, Cochain et al., 2012a, Berres et al., 2009, McKimmie et al., 2013, Lin et al., 2011, Di Liberto et al., 2008). However, with the exception of some initial findings, (McKimmie et al., 2008) there is little information about the factors that can regulate D6 expression. As D6 expression is regulated in psoriasis (See chapter 3), understanding which factors elevate D6 expression is important for a greater understanding of D6's role(s) in psoriasis.

The work described in this chapter examines the potential of inflammatory mediators to regulate D6 expression. Three different types of human cells were used to test the effect of inflammatory mediators in regulating D6 expression. As D6 immuno-reactivity was noted in the epidermis, LVs and PBMCs in this study, primary cell sources for each (Normal Human Epidermal Keratinocytes (NHEKs), and Human Dermal Lymphatic Endothelial Cells (HDLECs)) were obtained. PBMCs were isolated from 'buffy coats'. It should be noted here that all cytokine stimulations were performed at a concentration of 100ng/ml which is supraphysiological.

*In vivo* work, both in mice and humans, is also described in this chapter. Regulation of D6 expression in two mouse models of inflammation was investigated to determine whether the patterns of remote increases in D6 expression observed in clinical samples could be mirrored in mouse models of cutaneous inflammation. The first mouse model used was the

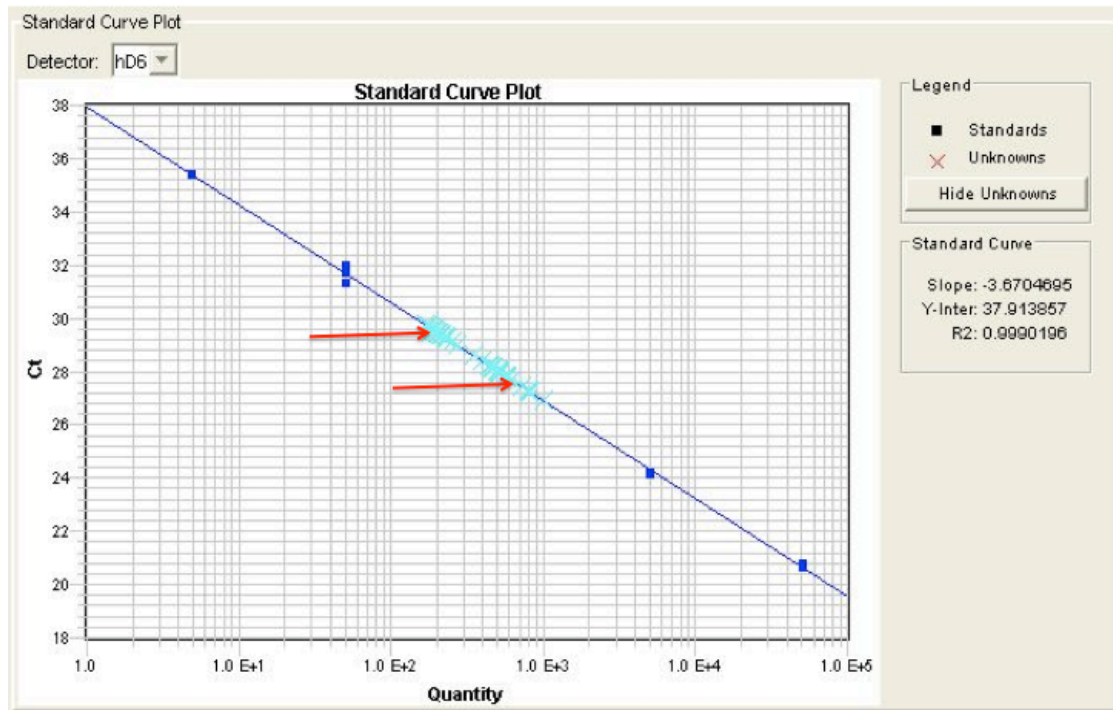
S100a7/a15 mouse model (Wolf et al., 2010). In this model, the proteins S100a7 (psoriasin) and S100a15 (koebnerisin) were over-expressed in the epidermis of the mouse. This resulted in “psoriasis priming”, as the resultant transgenic mice were susceptible to lesion formation upon a mild irritation/trauma. The second mouse model of psoriasis used was the Imiquimod mouse model of psoriasis (van der Fits et al., 2009). This model uses the TLR7 agonist Imiquimod, which was applied to the shaved dorsal skin every day for a week. This induces a psoriasiform pathology. Micro-trauma has been shown to have a role in the formation of new lesions, at sites that were previously uninvolved (Mak et al., 2009, Nestle et al., 2009b, Perera et al., 2012). Patient volunteers were therefore subjected to micro-trauma of the skin, then biopsies taken and D6 expression analysed. This was to examine whether micro-trauma could partially be responsible for the reduced D6 expression observed between uninvolved psoriatic skin, and peri-lesional psoriatic skin.

## **4.2. Effect of inflammatory mediators on keratinocyte D6 expression**

### **4.2.1. Examination of D6 expression in NHEKs**

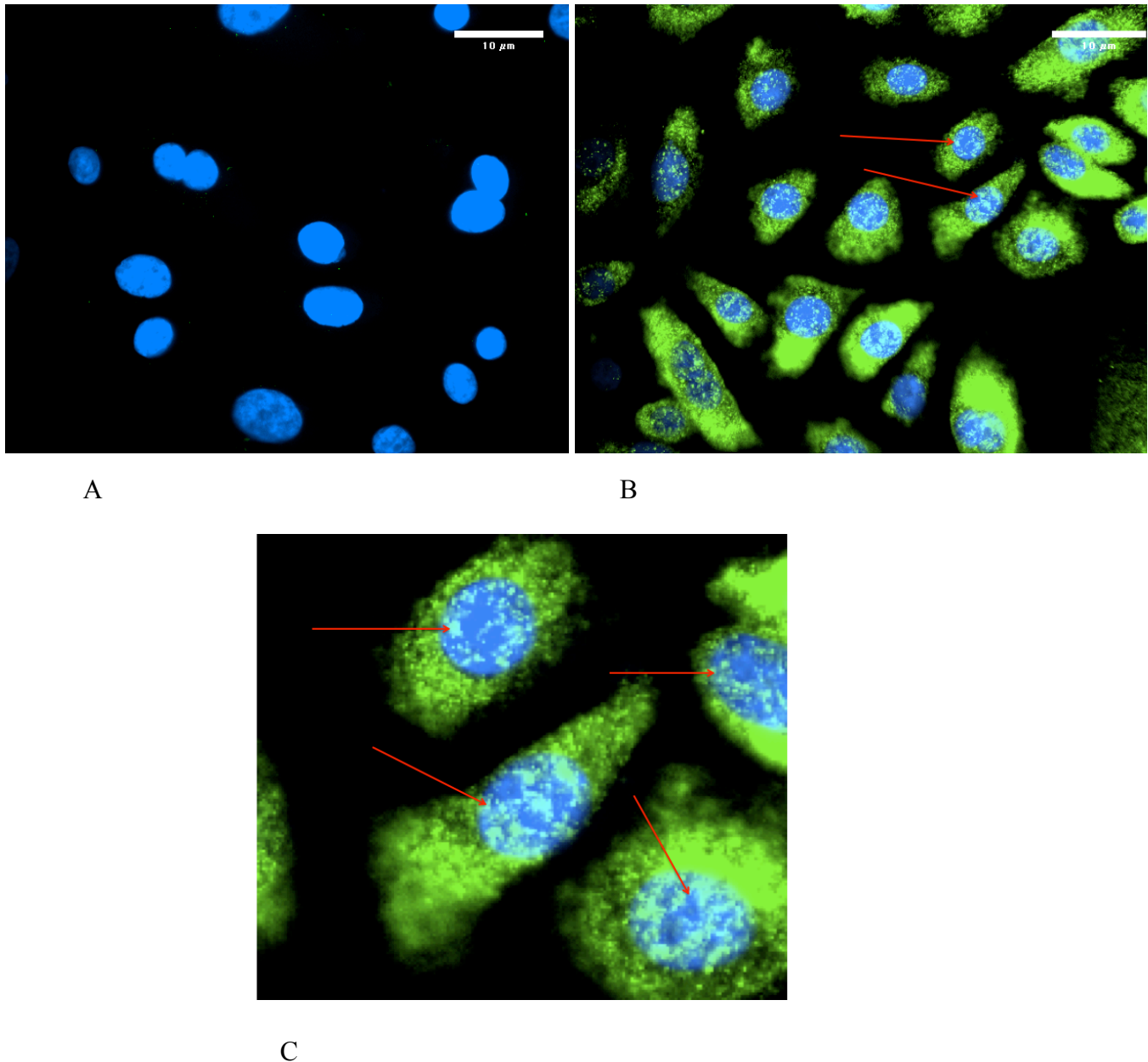
As detailed in chapter 3, the levels of D6 expression in the epidermis in psoriasis patients, and healthy controls, was markedly different. An *in vitro* system was therefore used to examine factor(s) which might be responsible for regulating D6 expression in epidermal keratinocytes. NHEKs were obtained to study the regulation of D6 expression in this cell type. NHEKs were seeded onto chambered slides, cultured until they were approximately 90-95% confluent, and then RNA extracted and purified. QPCR was then used to determine the number of D6 transcripts produced by NHEKs. Figure 4.1. is a standard curve generated during a QPCR run. The standard used is a serially diluted plasmid containing D6 cDNA. At a low number of passages, NHEK D6 is detectable between Ct (cycle threshold) values 27-29 (Figure 4.1., see red arrows). This is well within the detection limits of this assay. Thus, D6 expression in resting NHEKs can be detected by PCR.

To determine whether NHEKs also expressed D6 protein, they were seeded into 12-well plates, and cultured until 90-95% confluent. The cells were then fixed in 100% methanol and allowed to air dry. The cells were then stained for D6 (Sigma polyclonal antibody), and then imaged using IF microscopy (Figure 4.2.). No detectable IF staining was observed in NHEKs incubated with rabbit IgG fractions (Figure 4.2.A.). However, as shown in figure 4.2.B., D6 immuno-reactivity was detected on D6 antibody stained NHEKs. Importantly, punctate D6 staining is observed (Figure 4.2.C., highlighted by red



**Figure 4.1. D6 standard curve displaying the levels of NHEK-D6 expression.**

Red arrows point to the position D6 RNA is detected on the standard curve (Between cycle threshold (Ct) values of 27-29). This result is representative of 10 experiments.



**Figure 4.2. IF staining of D6 on NHEKs.**

NHEKs were stained with either A. Rabbit IgG fractions or B. D6 (Sigma) polyclonal antibody. Scale bars 10μm. Note the punctate D6-staining as highlighted by the red arrows. C. A cropped and enhanced image to more clearly show punctate staining (red arrows). These images are representative of at least 5 experiments.

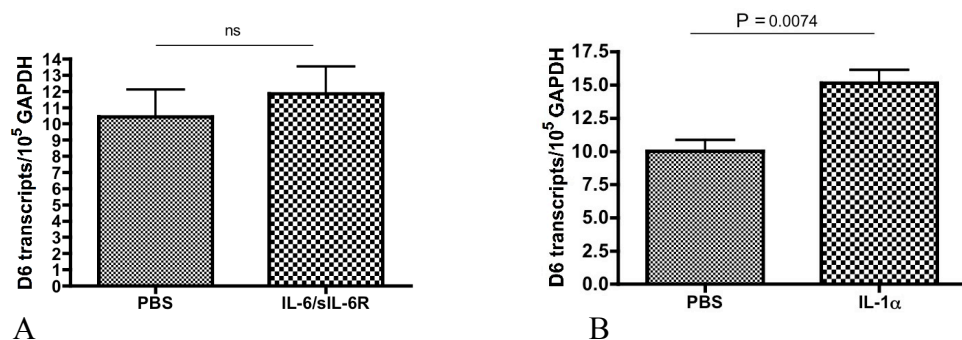


arrows). This punctate staining is characteristic of D6 expression *in vitro* as D6 is predominantly found within intracellular vesicles (Weber et al., 2004).

#### 4.2.2. Regulation of NHEK D6 expression by cytokines detected in uninvolved skin

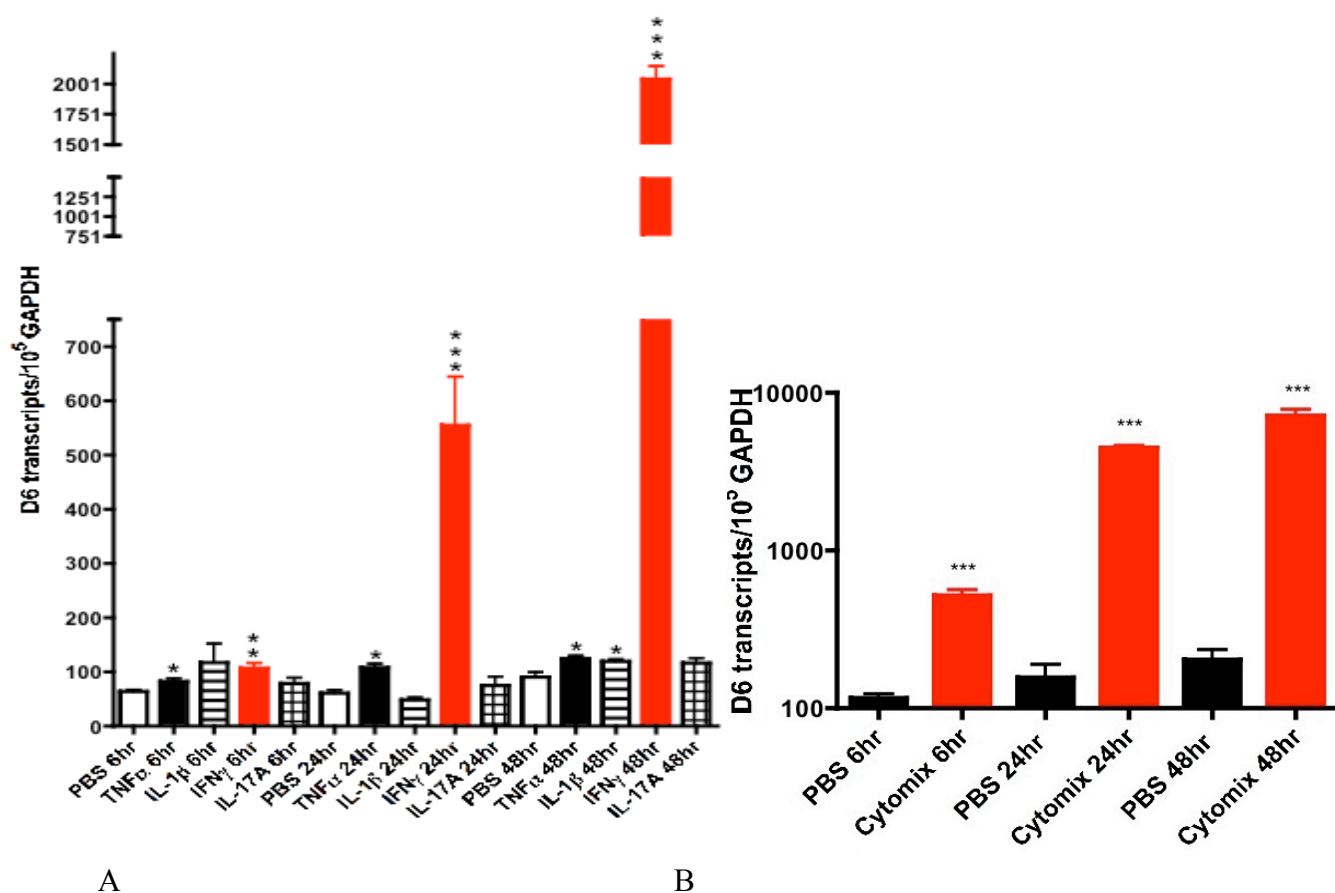
As D6 expression is found throughout the epidermal keratinocyte layers in uninvolved psoriatic skin (See chapter 3), the question of how D6 is regulated these cells was addressed. Uninvolved psoriatic skin is known to have on-going, low level inflammation (Kulski et al., 2005, Bowcock et al., 2001, Uyemura et al., 1993, Ohta et al., 1991, Yoshinaga et al., 1995). Microarray data has also been published showing marked differences in the transcriptional profile of uninvolved psoriatic skin compared to healthy control skin (Bowcock et al., 2001, Kulski et al., 2005). The gene alterations detected in these microarrays tend to be inflammatory in nature (Bowcock et al., 2001, Kulski et al., 2005). Moreover there are other studies using *in situ* hybridization that show marked elevation of the inflammatory cytokines IL-1 $\alpha$  and IL-6 within the stratum basale of uninvolved skin (Ohta et al., 1991). In addition, IFN $\gamma$ , TNF $\alpha$ , IL-1 $\alpha$  and IL-1 $\beta$  have been detected by PCR in uninvolved psoriatic skin (Uyemura et al., 1993)

To test the role of inflammatory mediators in D6 regulation at uninvolved sites, NHEKs were stimulated with a variety of inflammatory cytokines. While stimulating NHEKs with IL-6 and the soluble IL-6 receptor for 6 hours had no effect on D6 transcript levels (Figure 4.3.A.), a 6 hour stimulation of NHEKs with IL-1 $\alpha$  resulted in a modest, but significant, increase in D6 transcript numbers (Figure 4.3.B.). As TNF $\alpha$ , IL-1 $\beta$ , IL-17 and IFN $\gamma$  have been detected in uninvolved psoriatic skin as discussed earlier, NHEKs were stimulated with each of these cytokines. In addition, the number of time points tested was increased (6hrs as before, as well as 24 hours, and 48 hours of stimulation). NHEKs were also stimulated with a mixture of cytokines or “cytomix” (composed of TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$  and IL-17A), to examine whether the cytomix would elevate D6 expression synergistically.



**Figure 4.3. Stimulation of NHEKs with IL-6/sIL-6R or IL-1 $\alpha$ .**

NHEKs were stimulated with A. PBS or IL-6/sIL-6R or B. PBS or IL-1 $\alpha$ . NHEKs were stimulated with PBS or cytokines for 6 hours. Cytokines were used at 100ng/ml. Statistical significance was determined using an unpaired Student's t test. Figure 4.3. is representative of one of the two independent experiments. For each independent experiment, NHEKs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point.



**Figure 4.4. Stimulation of NHEKs with Th1/Th17 associated cytokines or cytomix.**

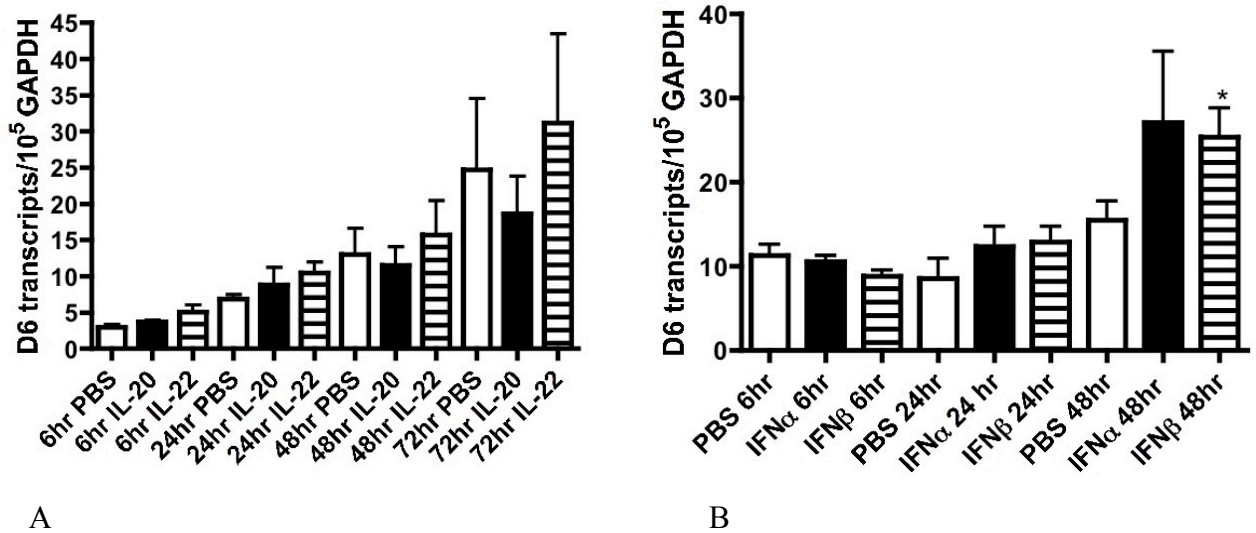
NHEKs were stimulated with A. PBS or TNF $\alpha$ , or IL-1 $\beta$ , or IL-17A, or IFN $\gamma$  for 6hrs, 24hrs or 48 hrs or B. PBS or cytomix (TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$ , IL-17A) Cytokines were used at 100ng/ml. Statistical analysis was determined using an unpaired Student's t test. \*P<0.03, \*\*P<0.01, \*\*\*P<0.0001. Figure 4.4. is representative of one of the two independent experiments. For each independent experiment, NHEKs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point.

Modest, but significant increases in D6 transcript numbers were noted when NHEKs were stimulated with TNF $\alpha$  at all time points, and IL-1 $\beta$  at later time points (Figure 4.4.A.). IL-17A did not regulate D6 expression levels at any of the time points tested (Figure 4.4.A.). Strikingly, IFN $\gamma$  induced a significant, 20-30 fold increase in the number of D6 transcripts after 24 and 48 hours of stimulation. (Figure 4.4.A.). These data suggest that while several inflammatory cytokines can elevate D6 expression, IFN $\gamma$  has a prominent role in elevating D6 expression in NHEKs. Admittedly, although multiple unpaired student's t test were used for statistical analysis, the author acknowledges that a one-way anova might have been a more appropriate test for multiple cytokine stimulation comparisons. Specifically, use of a one-way anova reduces the chance of spurious significant data being generated when comparing more than two groups. In addition, if Figures 4.3. and 4.4. are studied carefully, the numbers of D6 transcripts in PBS controls is markedly higher in Figure 4.4. compared to Figure 4.3. This is due to the passage number of the cells used. In our hands, D6 expression was found to decrease, as the number of passages increased (Singh and Graham unpublished observations). NHEKs used in Figure 4.3. had been passaged 6 times, compared to only two passages for NHEKs used in Figure 4.4. Stimulation of NHEKs with a cytomix induced a significant 40-fold increase in D6 transcript numbers after 24 hours stimulation (Figure 4.4.B.). Therefore it appears the other cytokines in the cytomix act in synergy with IFN $\gamma$  to induce even greater increases in the numbers of D6 transcripts. From these data, it is clear D6 that expression can be increased following inflammatory cytokine stimulation.

#### 4.2.3. Regulation of NHEK D6 expression by cytokines detected in lesional skin

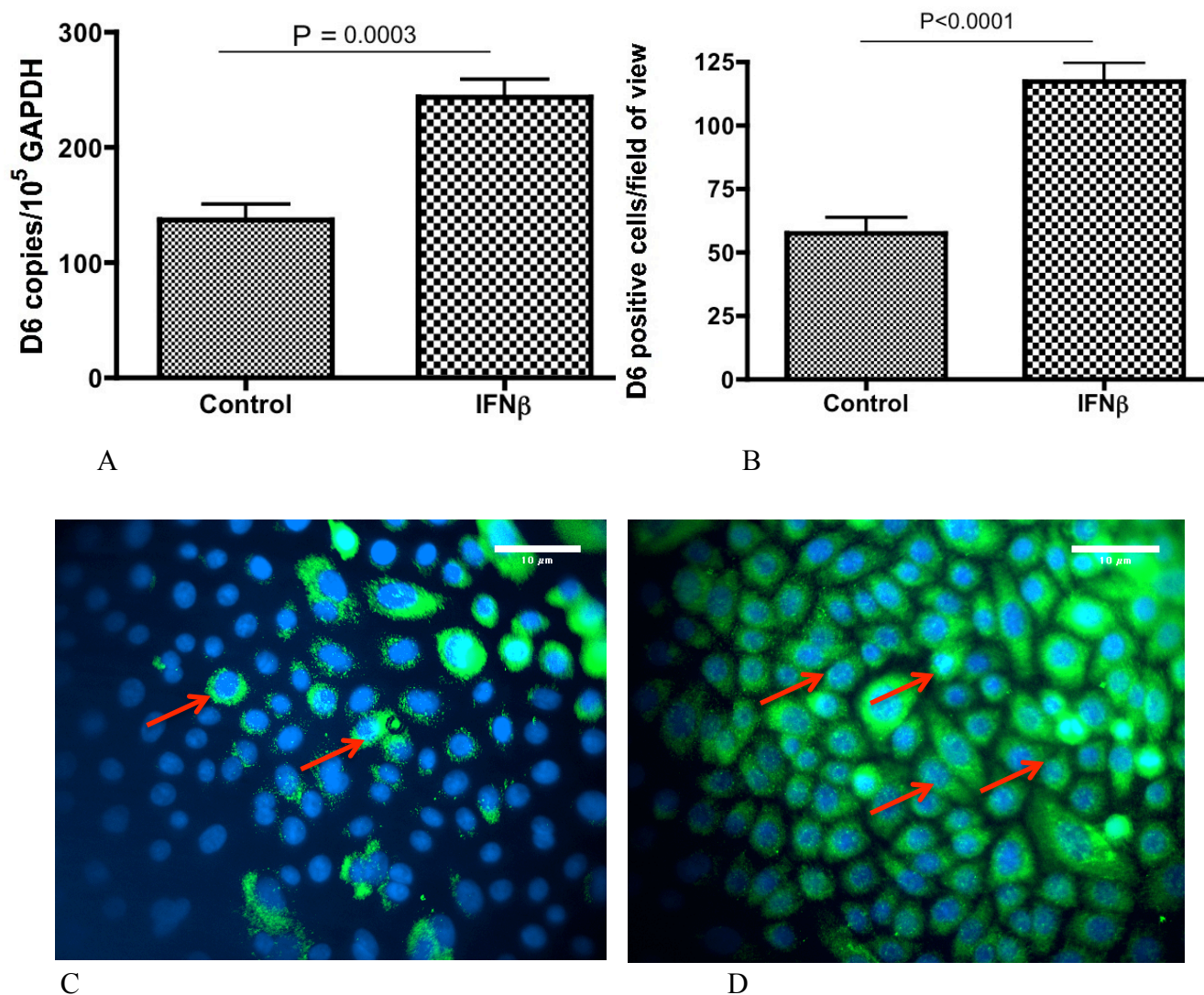
While some cytokines are found in both uninvolved psoriatic skin, and lesional psoriatic skin (such as the cytokines analysed in section 4.2.2.), there are other cytokines found in lesional psoriatic skin, but not uninvolved psoriatic skin. These include members of the IL-10 superfamily (IL-20 and IL-22), and IFN $\alpha$  and IFN $\beta$  (although reports are conflicting on whether type I IFNs are detectable in uninvolved psoriatic skin, see section 1.6.4.). Therefore, NHEKs were treated with these cytokines to examine whether they could also regulate D6 transcript numbers. Figure 4.5.A. shows that neither IL-20 nor IL-22 increased the levels of D6 RNA as measured by QPCR. Recently there has been interest in the role of pDCs in the initiation of psoriasis, particularly the role of IFN $\alpha$  (Nestle et al., 2005). pDCs are known to produce significant quantities of type I IFNs (IFN $\alpha$  and IFN $\beta$ ) and are present in psoriasis lesions. Therefore, the effect of stimulating NHEKs with Type I IFNs was tested. Although stimulation of NHEKs with IFN $\alpha$  had no effect on the expression of

D6, IFN $\beta$  induced a significant increase in D6 transcript levels 48 hours post stimulation (Figure 4.5.B.). A significant increase in D6 transcript numbers (Figure 4.6A) and protein (Figure 4.6.B.) was noted at 48 hours post stimulation in subsequent experiments. Examples of PBS treated, and IFN $\beta$  treated, NHEKs stained for D6 are displayed in Figure 4.6.C. and 4.6.D. (Highlighted with red arrows). Thus, D6 expression can be dynamically regulated in NHEKs by cytokine stimulation.



**Figure 4.5. Stimulation of NHEKs with cytokines found in psoriasis lesions.**

NHEKs were stimulated with A. PBS or IL-20 or IL-22 for 6hrs, 24hrs, 48hrs or 72hrs B. Stimulation of NHEKs with PBS or IFNα or IFNβ for 6hrs, 24hrs or 48hrs. Statistical significance was determined using an unpaired student's t test. Cytokines were used at 100ng/ml. All experiments were repeated twice. The data presented in Figure 4.5. are examples of one of the two independent experiments. For each independent experiment, NHEKs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point.



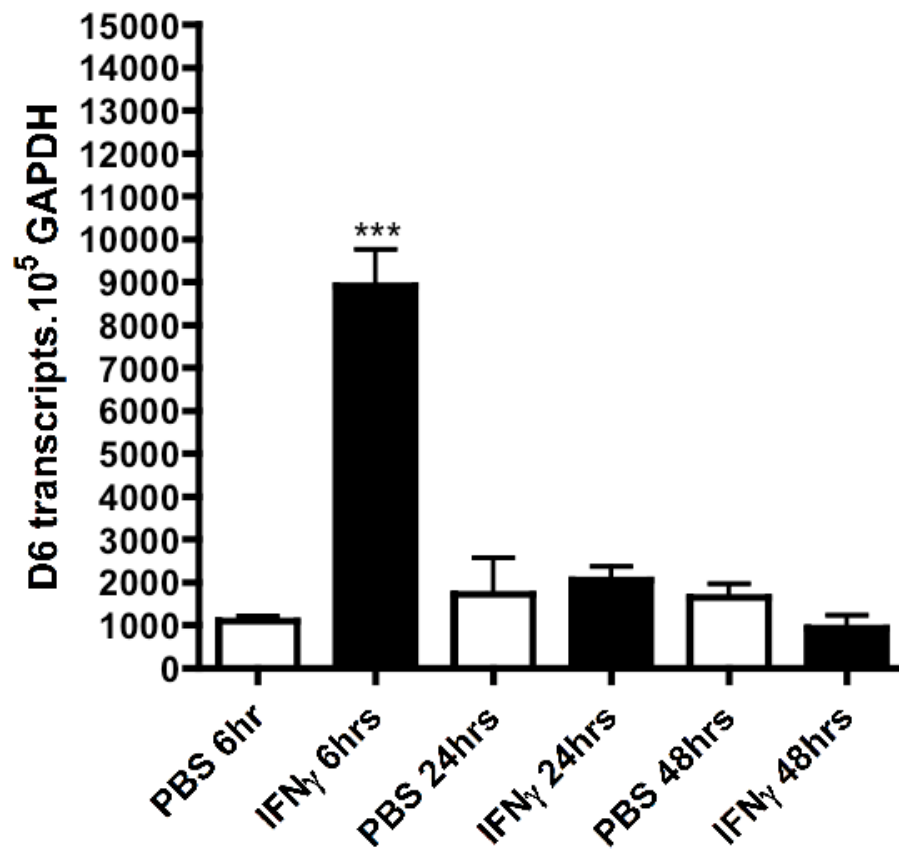
**Figure 4.6. Stimulation of NHEKs with IFN $\beta$  for 48 hrs.**

A. QPCR analysis of the number of D6 transcripts in PBS or IFN $\beta$  stimulated NHEKs or B. D6 immuno-stain counts in PBS or IFN $\beta$  stimulated NHEKs. IFN $\beta$  was used at 100ng/ml. D6 positive cells were enumerated by taking 10 different fields of view in PBS or IFN $\beta$  stimulated NHEKs, and counting the D6 positive cells. C. PBS stimulated NHEKs, stained with D6 Sigma antibody. D. IFN $\beta$  stimulated NHEKs, stained with D6 Sigma antibody. Statistical analysis was performed using an unpaired Student's t test. The data in Figure 4.6. is representative of 1 of 5 independent experiments For each independent experiment, NHEKs were seeded into multiple 4 well plates, and 4 wells were stimulated with either PBS, or cytokine, per time point. Scale bars 10 $\mu$ m.

### **4.3. Effect of inflammatory mediators on Human Dermal Lymphatic Endothelial Cell (HDLEC) D6 expression**

LVs as discussed previously, are established sites of D6 expression (Nibbs et al., 2001). In this study, it has been shown that the percentage-D6 positive LVs is elevated in uninvolved psoriatic skin compared to healthy controls, and peri-lesional, and lesional tissue (Figures 3.20. and 3.22.). This suggests that D6 expression on LVs, similar to epidermal keratinocytes, can be regulated by various factors. To determine whether D6 on LVs could be regulated by inflammatory cytokines, Human Dermal Lymphatic Endothelial Cells (HDLECs) were obtained from commercial sources and treated with inflammatory mediators. IFN $\gamma$  induced a significant, approximately 9 fold elevation of D6 expression in HDLECs (Figure 4.7.). Unlike NHEKs stimulated with IFN $\gamma$ , where the fold change increase in D6 expression increased with the length of the cytokine stimulation (Figure 4.4.A.), the increase in D6 transcript numbers by IFN $\gamma$  in HDLECs was a transient effect, with no difference in D6 expression observed after 24 hours, or 48 hours stimulation with PBS or IFN $\gamma$  (Figure 4.7.).

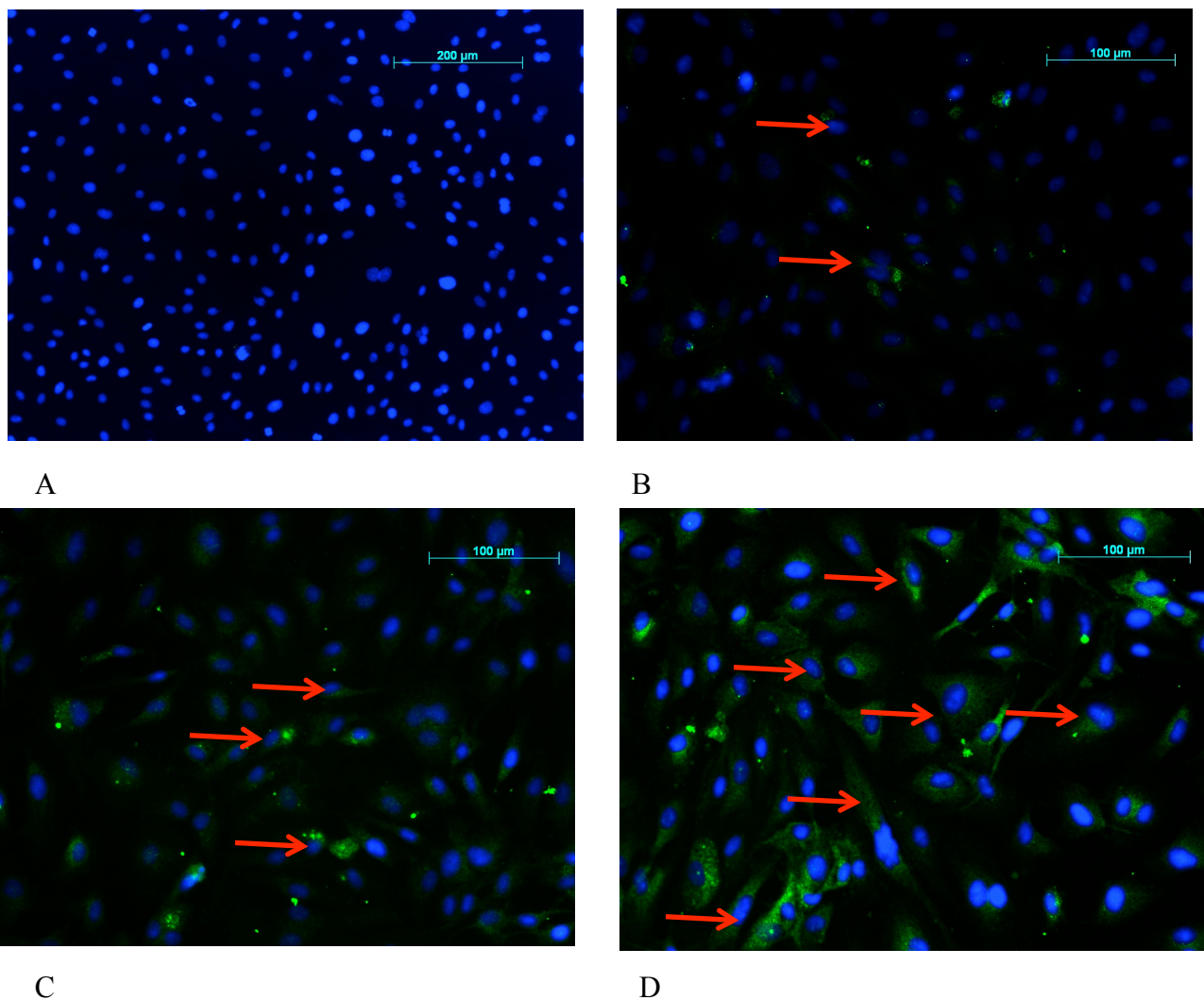
Other studies running concurrently with this work also demonstrated that IL-6 in combination with its soluble receptor, induced a significant 8-fold elevation of D6 transcript levels in HDLECs (McKimmie et al., 2013). Stimulation of HDLECs with IFN $\gamma$  and IL-6/sIL-6R also resulted in an increase in the levels of D6 protein (Figure 4.8.). No immuno-reactivity was detected in PBS stimulated HDLECs incubated with rabbit IgG fractions (Figure 4.8.A.). The immuno-stains of HDLECs stimulated with PBS (Figure 4.8.B.), IFN $\gamma$  (Figure 4.8.C.) or IL-6/sIL-6R (Figure 4.8.D.) are shown in Figure 4.8. (highlighted with red arrows) Figure 4.9. displays the quantification of the increases in D6 protein expression in HDLEC stimulated with IFN $\gamma$  (Figure 4.9.A.) or IL-6/sIL-6R (Figure 4.9.B.). As both IFN $\gamma$  and IL-6 are found in uninvolved psoriatic skin (Ohta et al., 1991, Uyemura et al., 1993), it is therefore possible that these mediators contribute significantly to the increased D6 expression on LVs described previously (Figures 3.20. and 3.22.).



**Figure 4.7. Stimulation of HDLECs with IFN<sub>γ</sub>.**

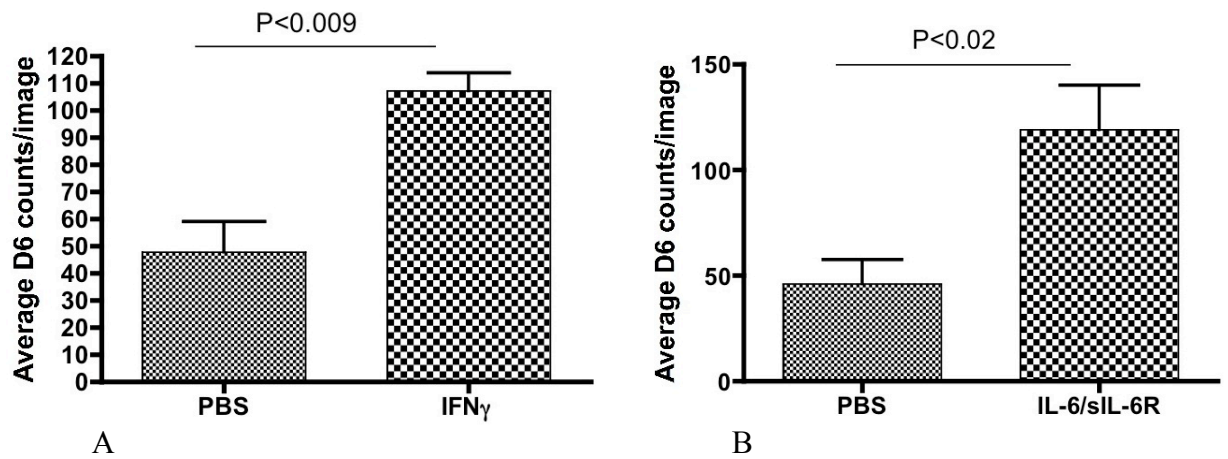
QPCR analysis of D6 transcript numbers in HDLEC stimulated with either PBS or IFN<sub>γ</sub> for 6hrs, or 24hrs or 48hrs. IFN<sub>γ</sub> was used at 100ng/ml. Statistical analysis was performed using unpaired Student's t test. \*\*\* P = 0.0006. The data in Figure 4.7. is representative of 1 of 2 independent experiments For each independent experiment, HDLECs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point.





**Figure 4.8. D6 immuno-staining of HDLECs stimulated with IFN $\gamma$  or IL-6/sIL-6R.**

A. PBS stimulated HDLECs, incubated with Rabbit IgG fraction. B. PBS stimulated HDLECs, D6 immuno-staining. C. IFN $\gamma$  stimulated HDLECs, D6 immuno-staining. D. IL-6/sIL-6R stimulated HDLECs, D6 immuno-staining. D6 Immunostaining was performed on PBS or IFN $\gamma$  or IL-6/sIL-6R stimulated HDLECs after 24 hours cytokine stimulation. Cytokines were used at 100ng/ml. Red arrows indicate positive D6-staining. Scale bar A - 200 $\mu$ m, scale bars B-D - 100 $\mu$ m. The D6 immunostaining in Figure 4.8. are representative of 1 of 3 independent experiments. For each independent experiment, HDLECs were seeded into multiple well plates, and 4 wells were stimulated with either PBS, or cytokine, per time point.



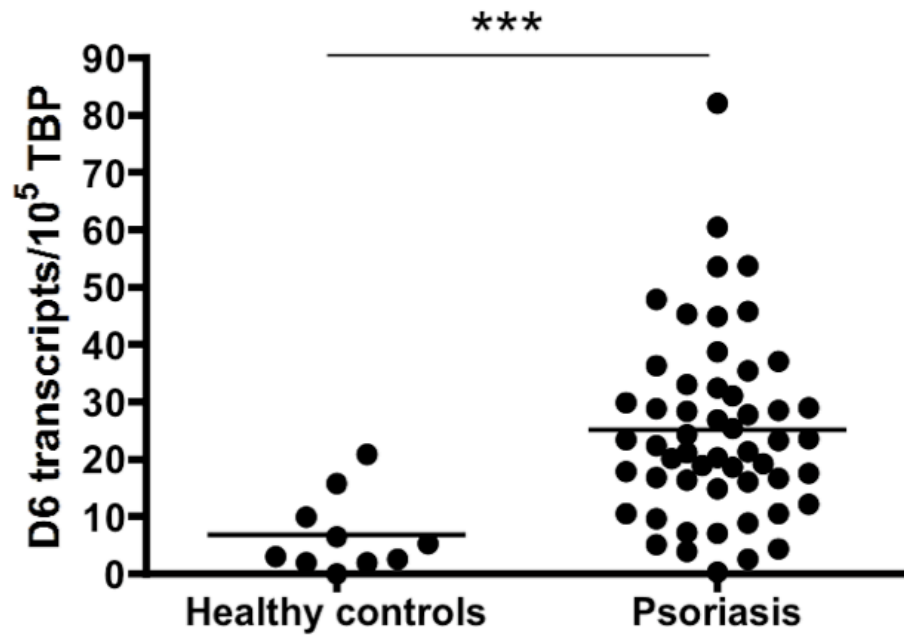
**Figure 4.9. Enumeration of D6-positive HDLECs stimulated with inflammatory cytokines.**

HDLECs were stimulated with A. PBS or IFN $\gamma$  for 24 hours, or B. PBS or IL-6/sIL-R for 24 hours. D6 positive-cells were calculated by counting 5 views of each imaged slide, and taking the average number. The data in Figure 4.9. is representative of 1 of 3 independent experiments For each independent experiment, HDLECs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point. Statistical analysis was performed using an unpaired student's t test.

#### **4.4. Correlation of PBMC D6 transcripts with mediators of inflammation**

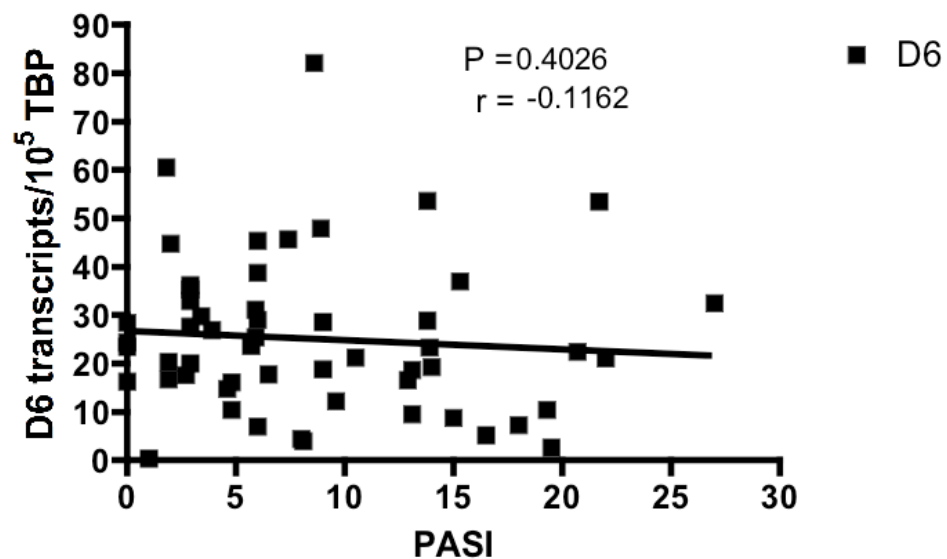
If figure 3.29. is examined closely, it is clear the numbers of D6 transcripts produced by psoriasis PBMCs are variable, ranging from just less than 1 transcript, up to 100 D6 transcripts per 100,000 copies of the housekeeping gene (TBP), suggesting that PBMC-D6 expression is regulated by some unknown factor(s). In an attempt to identify what factor(s) could be increasing PBMC-D6 expression in psoriasis PBMCs, a second larger cohort of psoriasis PBMCs was obtained, in addition to healthy controls. The RNA was then extracted and purified, and D6 expression measured by QPCR as before. In addition, a plasma sample was taken from each psoriasis patient and healthy control for luminex analysis of inflammatory mediators. As before, a significant increase in the numbers of D6 transcripts in psoriasis PBMCs compared with healthy control PBMCs was seen as shown in figure 4.10. Similar to the data in figure 3.29, a considerable spread in the D6 transcript levels is observed in the psoriasis cohort (Figure 4.10.).

With this notable variation in the numbers of D6 transcripts in the psoriasis cohort, we hypothesized that D6 expression may correlate with markers of disease severity. The marker used was the PASI score. The PASI score (Psoriasis Area Severity Index) is the standardized system to analyse the severity of disease in psoriasis (Marks et al., 1989). As shown in figure 4.11., there was no significant correlation between D6 transcript levels, and the PASI score. However, there appeared to be a general reduction in D6 expression as the PASI score increased.



**Figure 4.10. QPCR analysis of D6 transcript numbers in healthy control PBMCs and psoriasis PBMCs.**

Healthy control N = 10 Psoriasis N = 54. Statistical analysis was performed using a Mann Whitney U test. \*\*\* P<0.0001.

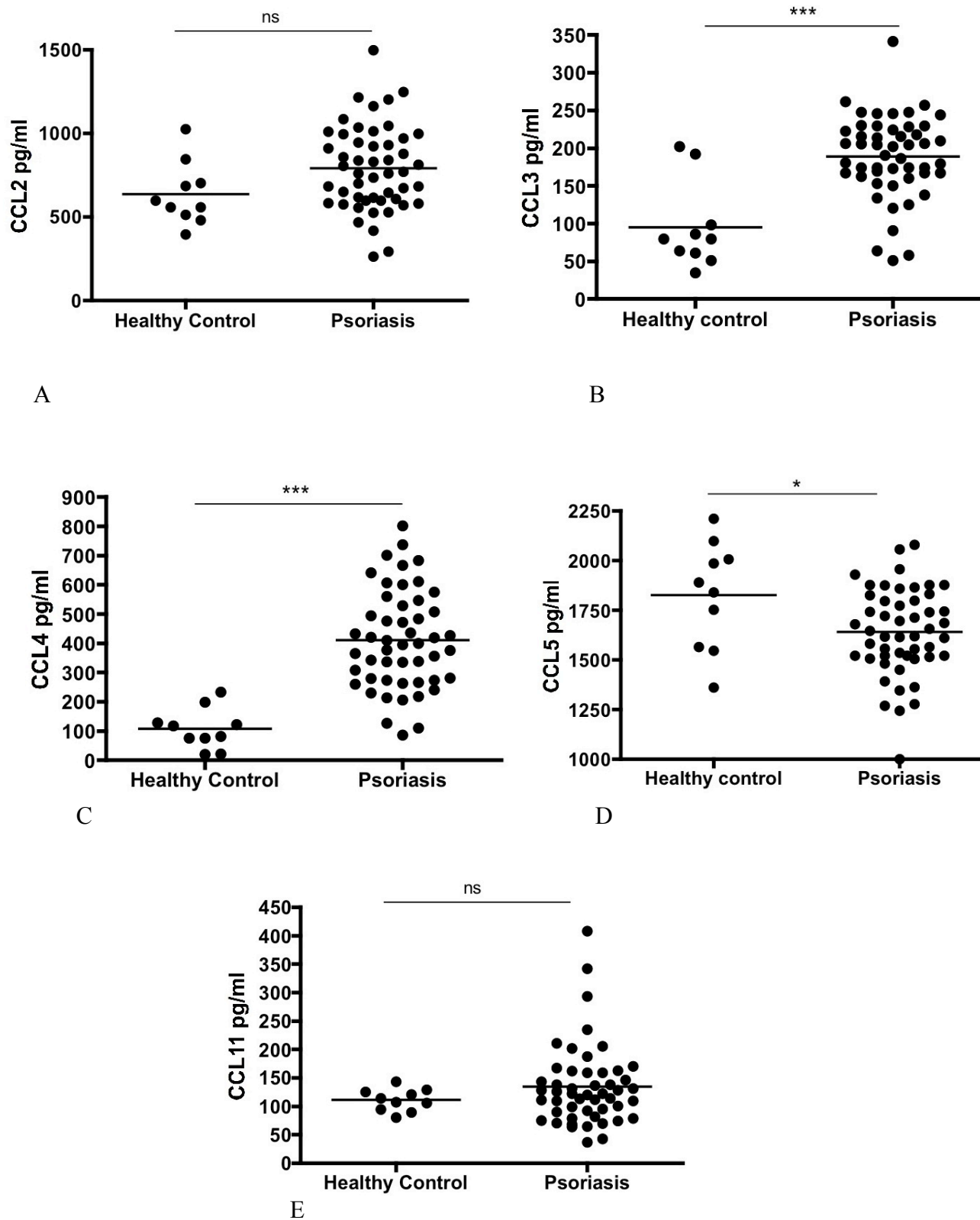


**Figure 4.11. Correlation analysis comparing PASI levels and D6 transcript numbers.**

Sample size N = 54. Statistical analysis was determined using a Spearman r test.

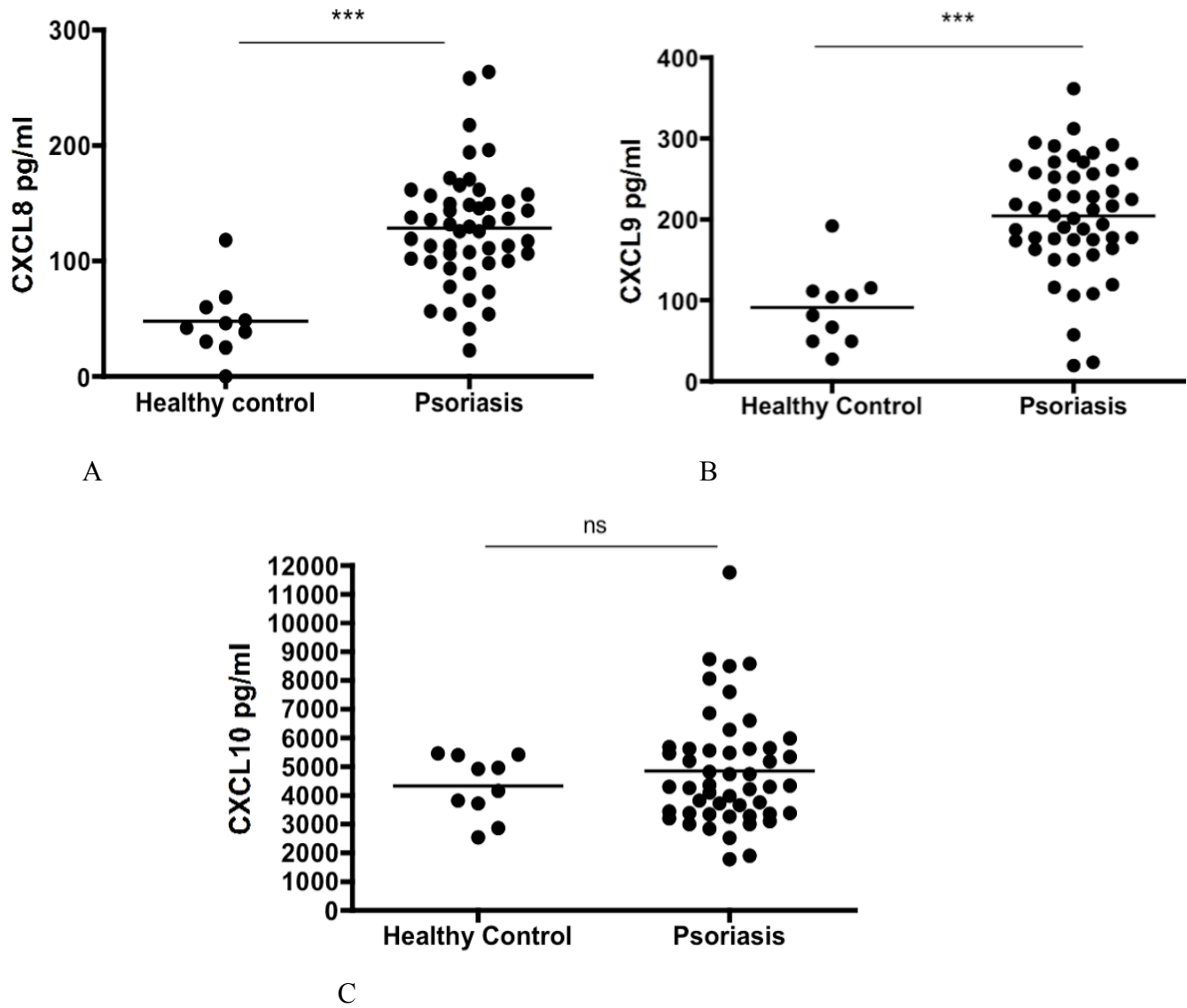
To gain further insights into the levels of circulating inflammatory mediators in healthy controls and psoriasis patients, a multiplex was undertaken. The luminex plate used allows the analysis of the levels of 30 different inflammatory mediators, allowing a wide range of mediators to be investigated simultaneously. No differences in the levels of CCL2, a high affinity D6 ligand was observed when comparing healthy control plasma and psoriasis plasma (Figure 4.12.A.) However, several of the inflammatory CC chemokines (D6 binding ligands) tested were increased in psoriasis plasma compared to healthy controls. CCL3 (Figure 4.12.B.) and CCL4 (Figure 4.12.C.) were significantly elevated in psoriasis plasma compared to healthy controls. Interestingly, CCL5 was modestly, but significantly reduced in psoriasis plasma compared to healthy control plasma (Figure 4.12.D.). No differences in the plasma levels of CCL11 were detected between healthy controls and psoriasis patients (Figure 4.12.E.). Analysis of the CXC chemokines detected in the multiplex revealed that both CXCL8 (Figure 4.13.A.) and CXCL9 (Figure 4.13.B.) were significantly elevated in psoriasis circulation compared with healthy controls. In contrast CXCL10 was not elevated in psoriasis circulation compared with healthy controls (Figure 4.13.C.).

Increased plasma levels of IFN $\alpha$  (Figure 4.14.A.), IFN $\gamma$  (Figure 4.14.B.) and IL-2 (Figure 4.14.C.) were observed in psoriasis plasma compared with healthy control plasma. IFN $\alpha$  has been shown to have a role in the initiation of psoriasis (Nestle et al., 2005), and IFN $\gamma$  is known to be involved in psoriasis pathogenesis (Uyemura et al., 1993). As psoriasis is a T-cell mediated disease, elevated levels of IL-2 are perhaps not unexpected. IL-1RA was significantly elevated in psoriasis plasma compared with healthy controls (Figure 4.14.D.). Levels of IL-1 $\beta$  however were similar in healthy control plasma and psoriasis plasma (Figure 4.14.E.) Interestingly, the Th2 cytokines IL-4 (Figure 4.15.A.), IL-5 (Figure 4.15.B.), IL-13 (Figure 4.15.C.) were also elevated in psoriasis plasma. As psoriasis is a Th1/17/22 mediated disease, elevation of Th2 cytokines is surprising. In addition, the anti-inflammatory cytokine IL-10 (Figure 4.15.D.), in addition to IL-7 (Figure 4.15.E.) were also elevated in psoriasis plasma compared to healthy controls. It is possible that elevated cytokine production, is a generalized feature of psoriasis. Elevated IL-6 (Figure 4.16.A), IL-15 (Figure 4.16.B.), IL-17 (Figure 4.16.C.) and TNF $\alpha$  (Figure 4.16.D.) in psoriasis plasma was perhaps to be expected, as they have all been reported to be involved in psoriasis (Perera et al., 2012, Michalak-Stoma et al., 2011). Figure 4.17. displays various growth factors that are all elevated in psoriasis plasma compared with healthy controls. Increased VEGF-A in psoriasis plasma compared to healthy control plasma (Figure 4.17.E.) is not surprising as VEGF-A is a known angiogenic factor,



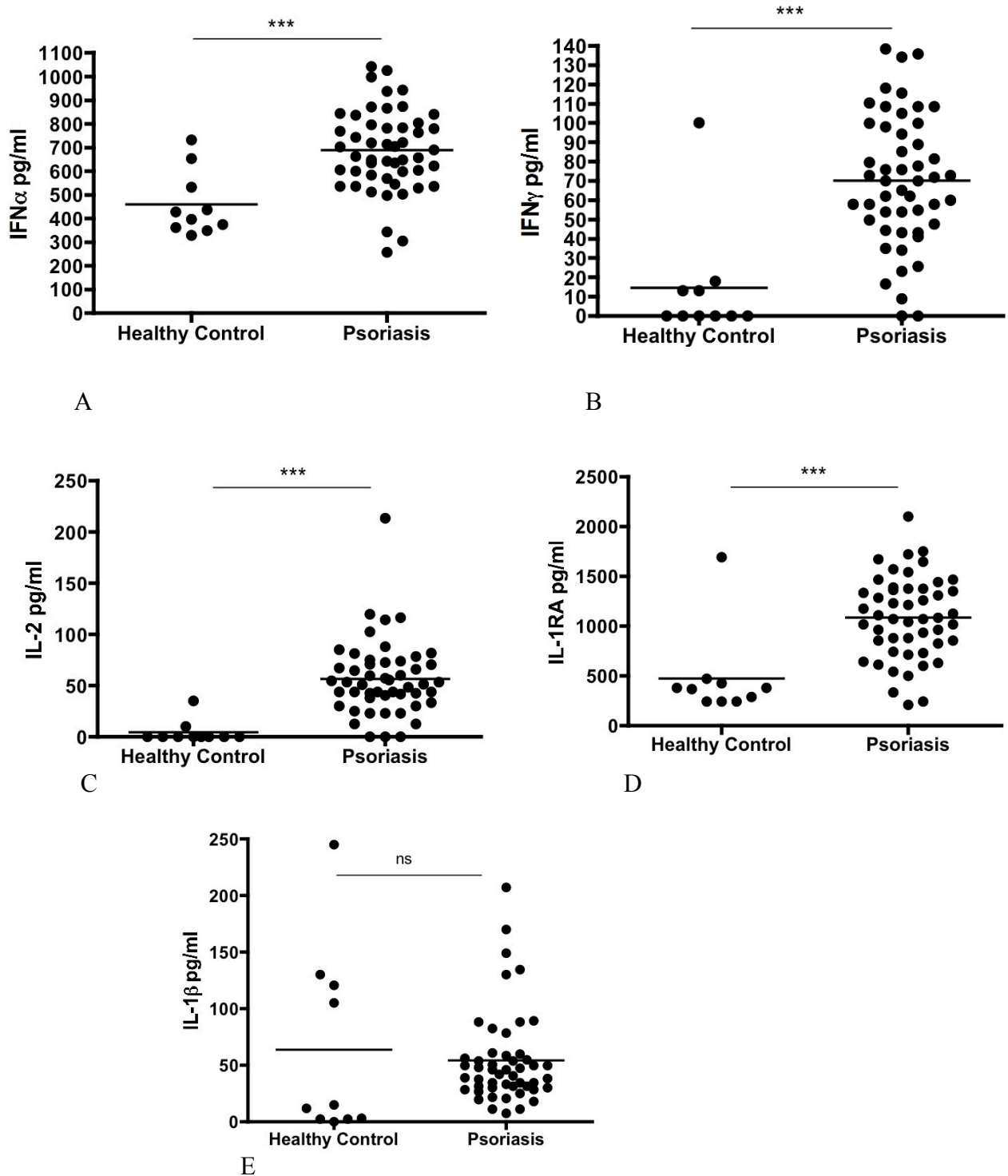
**Figure 4.12. Analysis of the levels of circulating inflammatory CC chemokines in healthy control plasma and psoriasis plasma.**

Healthy control N = 10, Psoriasis patients N = 54. Statistical analysis was performed using a Mann Whitney U test. \* P<0.03, \*\*\*P<0.0004.



**Figure 4.13. Analysis of the levels of circulating inflammatory CXC chemokines in healthy control plasma and psoriasis plasma.**

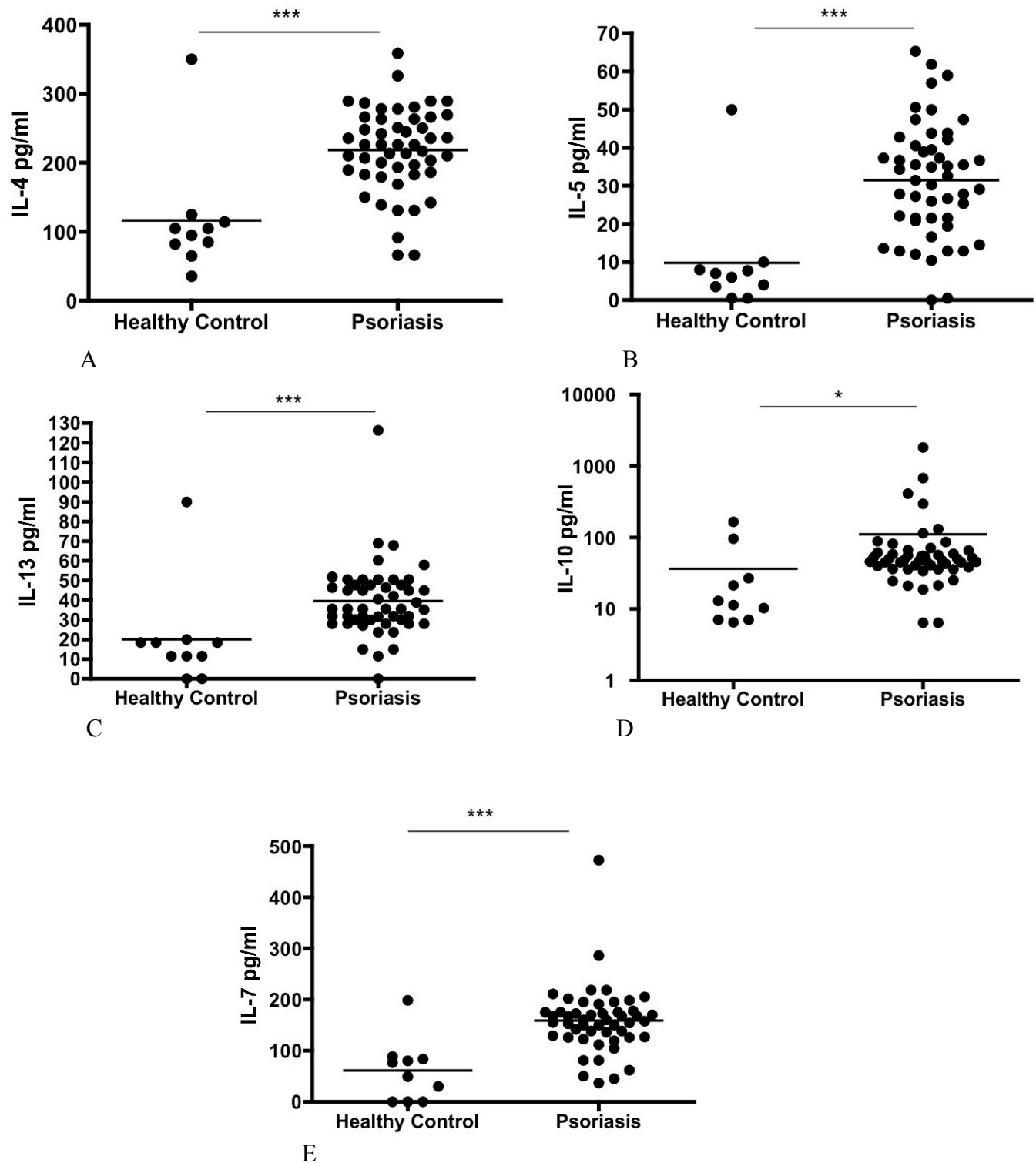
Healthy control N = 10, Psoriasis patients N = 54. Statistical analysis was performed using a Mann Whitney U test. \*\*\*P<0.0001.



**Figure 4.14. Analysis of the levels of circulating cytokines in healthy control plasma and psoriasis plasma I.**

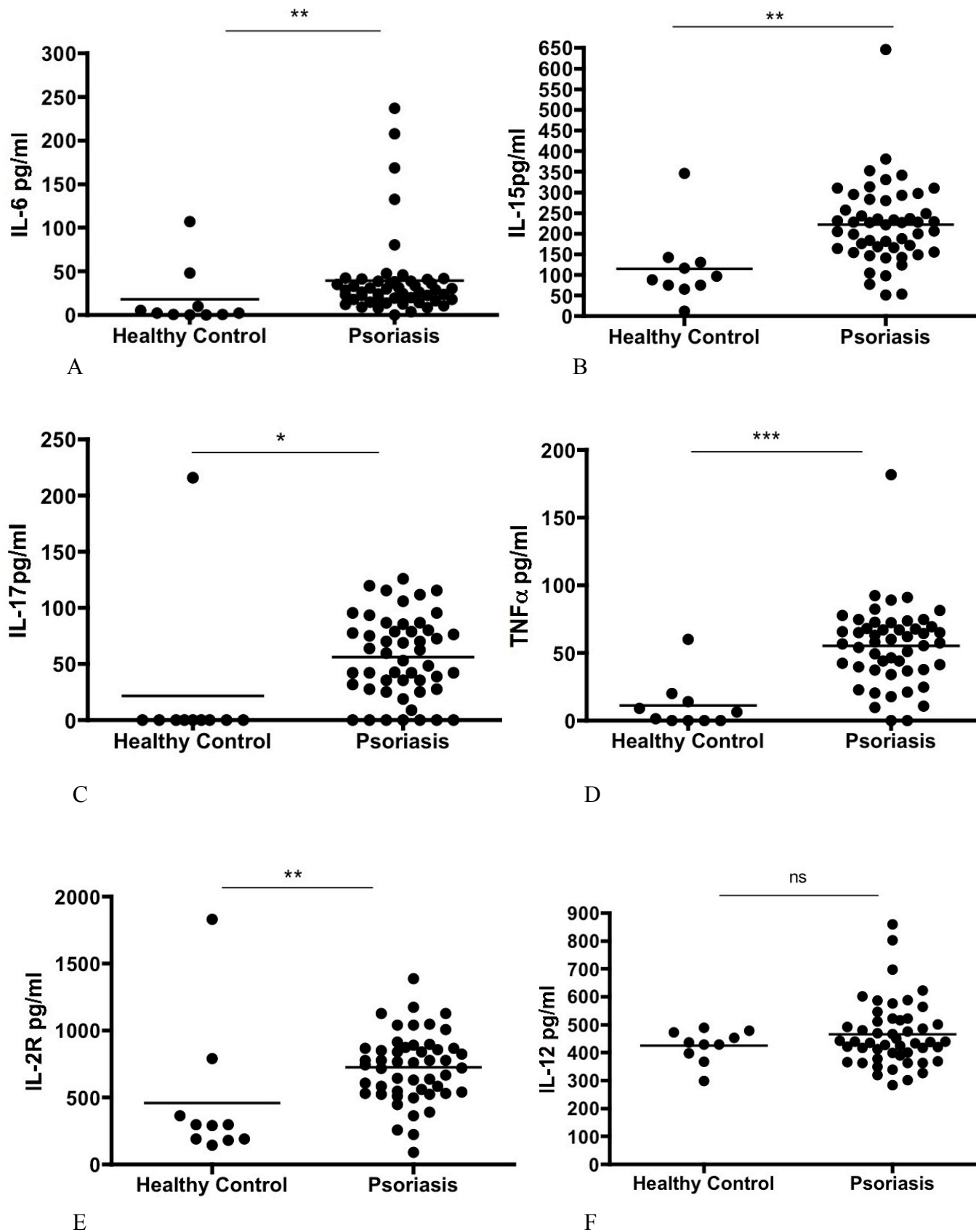
Healthy control N = 10, Psoriasis patients N = 54. Statistical analysis was performed using a Mann Whitney U test. \*\*\*P<0.0008.





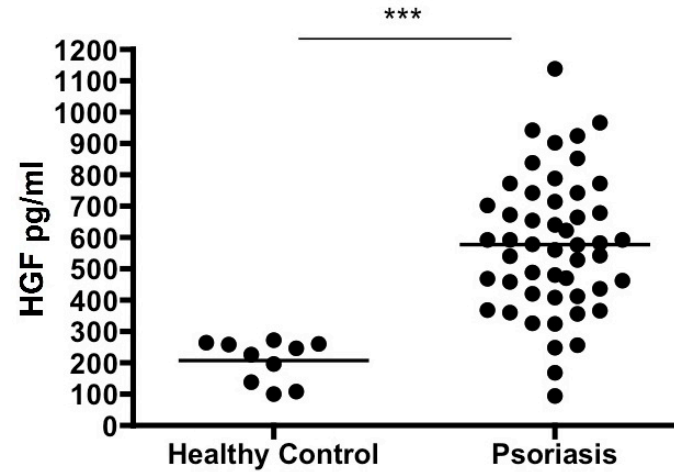
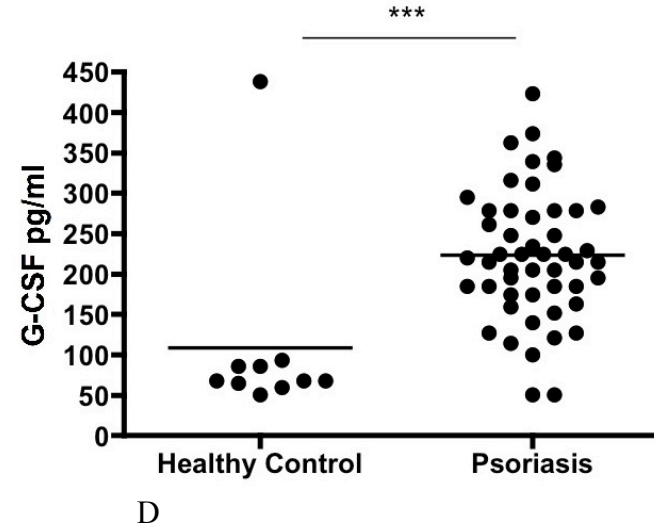
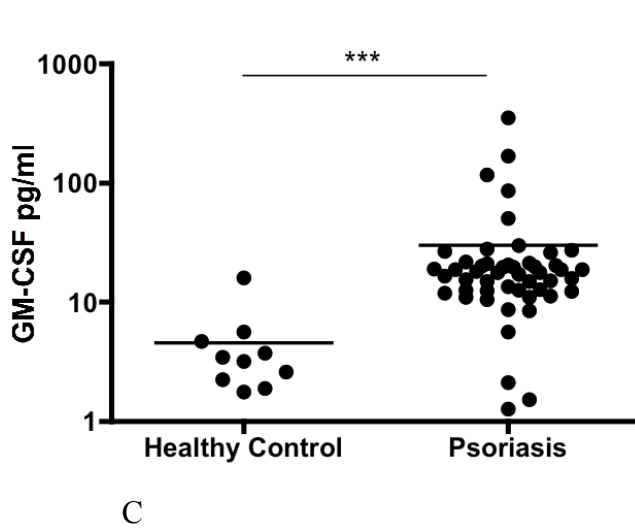
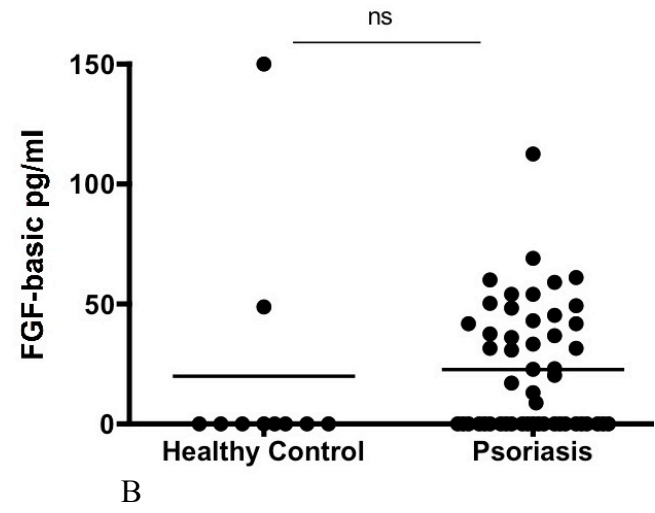
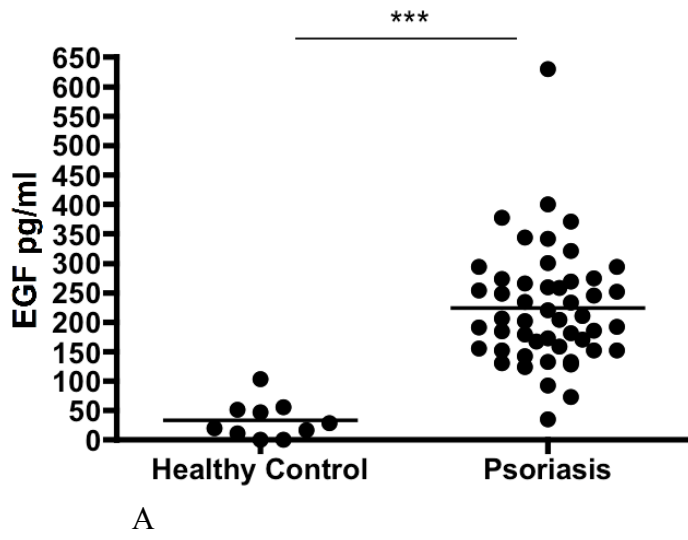
**Figure 4.15. Analysis of the levels of circulating cytokines in healthy control plasma and psoriasis plasma II.**

Healthy control N = 10, Psoriasis patients N = 54. Statistical analysis was performed using a Mann Whitney U test. \*\*\*P<0.0002, \*P<0.007.



**Figure 4.16. Analysis of the levels of circulating cytokines in healthy control plasma and psoriasis plasma III.**

Healthy control N = 10, Psoriasis patients N = 54. Statistical analysis was performed using a Mann Whitney U test. \*P<0.03, \*\*P<0.003, \*\*\*P<0.0001.

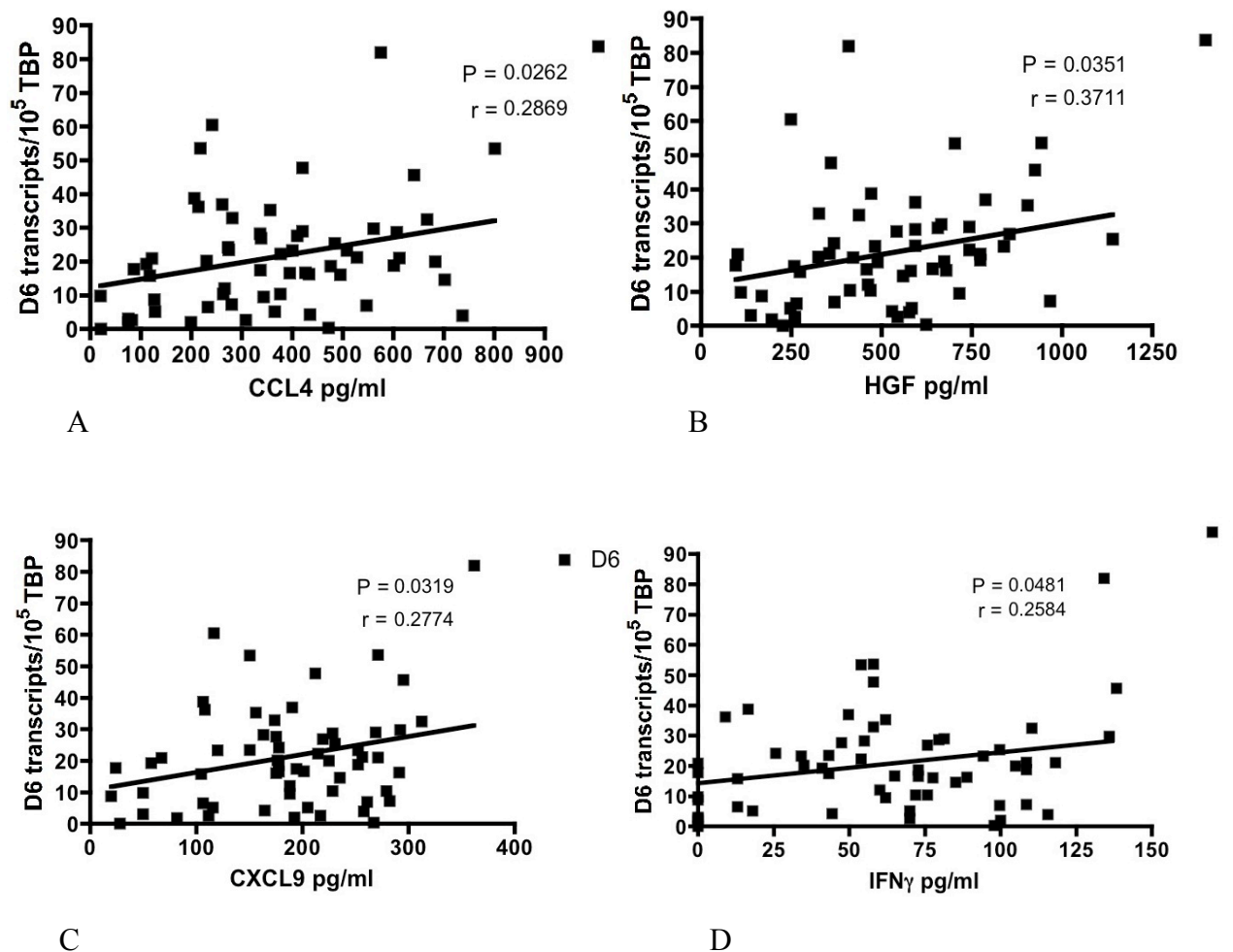


**Figure 4.17. Analysis of the levels of circulating cytokines in healthy control plasma and psoriasis plasma IV.**

Healthy control N = 10, Psoriasis patients N = 54. Statistical analysis was performed using a Mann Whitney U test. \*\*P<0.005, \*\*\*P<0.0001.

and increased angiogenesis has been reported in psoriasis (Canavese et al., 2010).HGF is also significantly elevated in psoriasis plasma compared to healthy control plasma (Figure 4.17.F.). HGF has been reported to be involved in wound healing, and as psoriasis has been described as aberrant wound healing (Perera et al., 2012), increased HGF is logical in this context.

To examine whether PBMC D6 transcript numbers correlate with any of the inflammatory mediators examined in the luminex, a correlation analysis was undertaken. As shown in figure 4.18., the numbers of D6 transcripts were found to positively correlate with plasma levels of CCL4 (Figure 4.18.A.), HGF (Figure 4.18.B.), and CXCL9 (Figure 4.18.C). CCL4 is of obvious importance, as it is a high affinity D6-binding ligand (Graham, 2009). HGF is a growth factor that has been reported to have a role in wound healing as discussed previously. Increased levels of circulating CXCL9 are also of interest, as elevated CXCL9 expression is detected in psoriasis lesions, and has been reported to be one of the chemokines involved in the migration of T-cells into the lesions (Flier et al., 2001). IFN $\gamma$  also positively correlated with D6-PBMC expression levels (Figure 4.18.D.) As IFN $\gamma$  can regulate D6 expression on NHEKs and HDLECs (see sections), it is possible that a similar effect also occurs in PBMCs. This is examined in section 4.5. To sum up this section, these data show that elevated PBMC-D6 expression is associated with elevated inflammatory mediators in the plasma. This suggests a role for the increased inflammatory mediators in increasing D6 expression at this site.



**Figure 4.18. Positive correlations between PBMC D6 transcript numbers and inflammatory mediators.**

A. CCL4, B. HGF, C CXCL9, D. IFN $\gamma$ . Sample size  $N = 64$ . Concentrations of inflammatory mediators are in pg/ml. Statistical significance was determined using a Spearman  $r$  test.

## **4.5. Effect of inflammatory mediators on human peripheral blood mononuclear cells (PBMCs) D6 expression**

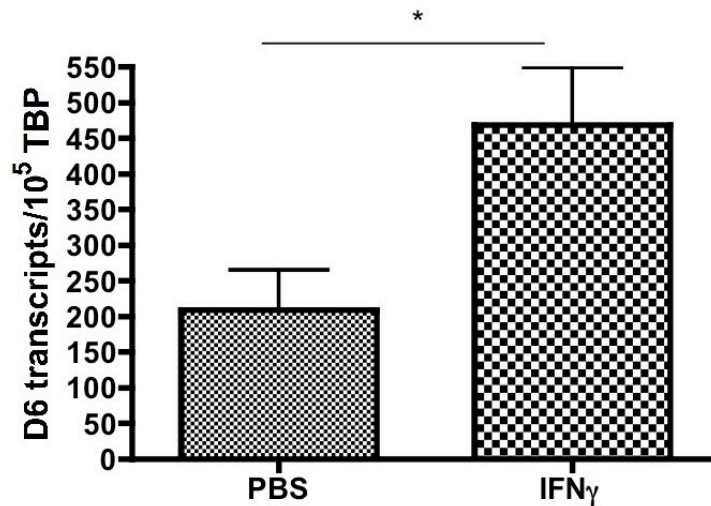
### **4.5.1. Effect of Interferons on PBMC-D6 expression**

As shown previously (Figure 3.29. and Figure 4.10.) there is a significant increase in the number of D6 transcripts in circulating PBMCs in psoriasis patients compared with healthy controls. To test if PBMC-D6 can be regulated by inflammatory cytokines, PBMCs were isolated from buffy coats using ficoll mediated separation as detailed in the Materials and Methods section. The PBMCs were then seeded into 12-well plates, then stimulated with various inflammatory cytokines. As plasma concentrations of IFN $\gamma$  were found to positively correlate with PBMC-D6 expression (Figure 4.18.D.), PBMCs were stimulated with IFN $\gamma$ . As shown in Figure 4.19., IFN $\gamma$  stimulation of PBMCs induces a modest, but significant increase in D6 expression.

IFN $\beta$  was shown earlier (section 4.2) to significantly elevate D6 expression both at the RNA and protein level in NHEKs (Figure 4.6.) In another study in the group, IFN $\beta$  also induced a two fold increase in D6 transcript numbers in HDLECs (McKimmie et al., 2013). Therefore PBMCs were stimulated with either IFN $\alpha$ , IFN $\beta$  or both IFN $\alpha$  and IFN $\beta$  to determine whether or not these cytokines could elevate D6 expression on PBMCs. Figure 4.20. shows that neither of the type I IFNs on their own, or in combination, resulted in an elevation in the number of PBMC D6 transcripts. It should be noted here that the reason for the difference in D6 expression in PBS stimulated PBMCs in Figure 4.19. compared to Figures 4.20.-4.25. is due to the fact that increased numbers of PBMCs were used in Figure 4.19. (20 million per stimulation) compared with Figures 4.20.-4.25. (2 million per stimulation).

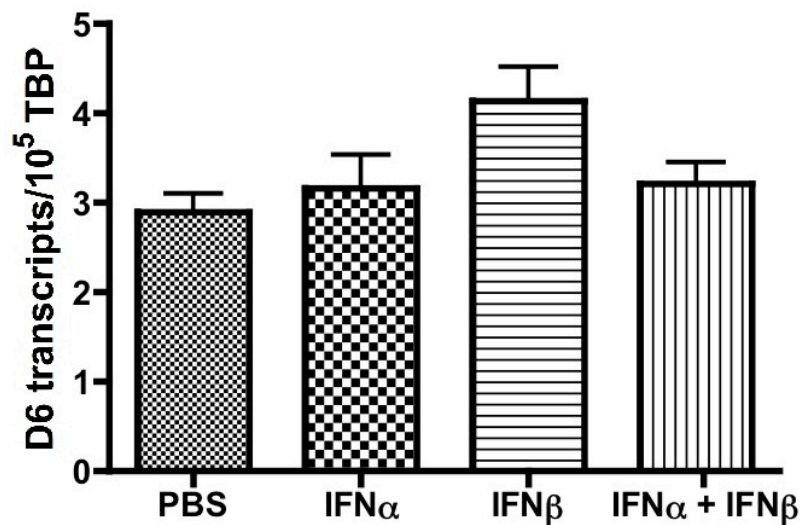
### **4.5.2. Effect of Interleukins on PBMC-D6 expression**

To examine whether other inflammatory mediators could induce a more potent up-regulation of D6 expression in PBMCs, a variety of interleukins, and TNF $\alpha$  were used to stimulate PBMCs, then D6 expression was analysed. While none of the cytokines tested induced a significant elevation in the levels of D6 expression in PBMCs, following IL-17 stimulation, there appeared to be a modest, but non-significant increase in D6 transcript numbers (Figure 4.21.). It was possible the time-point examined was either too early, or too late to observe any significant increase (or indeed decrease) in the numbers of D6 transcripts.



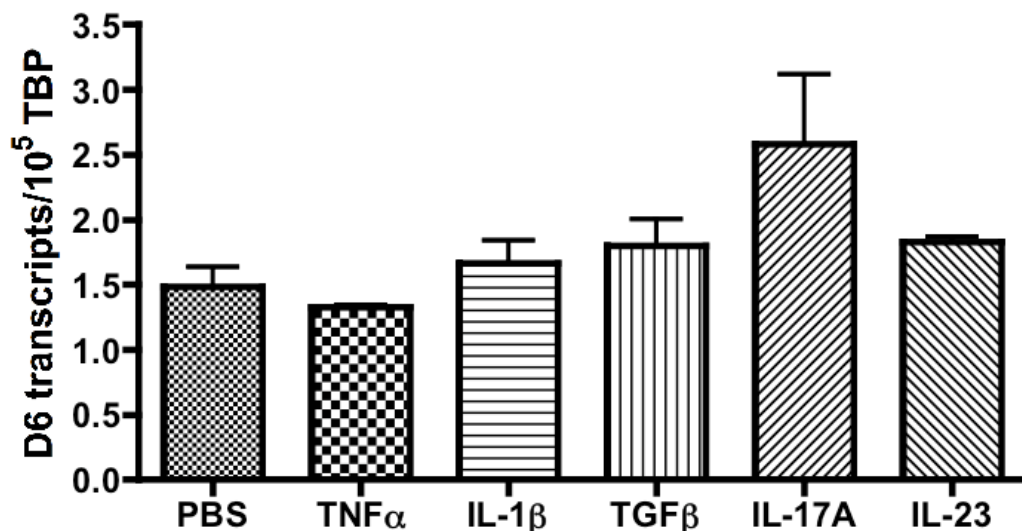
**Figure 4.19. QPCR analysis of D6 transcript numbers in IFN<sub>γ</sub> stimulated PBMCs.**

PBMCs were stimulated with either PBS or IFN<sub>γ</sub> for 6 hours. Cytokines were used at 100ng/ml. Statistical significance was determined using an unpaired student's t test. Figure 4.19. is representative of 1 of 4 independent experiments For each independent experiment, PBMCs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point.



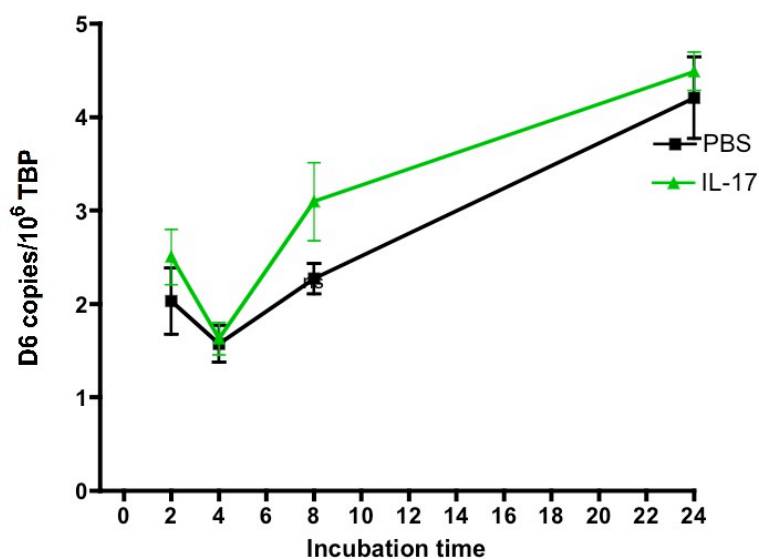
**Figure 4.20. QPCR analysis of D6 transcript numbers in PBMCs stimulated with type I IFNs.**

PBMCs were stimulated with either or IFN<sub>α</sub>, or IFN<sub>β</sub>, or a combination of IFN<sub>α</sub> and IFN<sub>β</sub>. Stimulations were performed for 6 hours. All cytokines were used at a concentration of 100ng/ml. Figure 4.20. is representative of 1 of 2 independent experiments For each independent experiment, PBMCs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point. Statistical significance was determined using an unpaired student's t test.



**Figure 4.21. QPCR analysis of D6 transcript numbers in PBMCs stimulated with inflammatory cytokines.**

PBMCs were stimulated with either TNF $\alpha$ , or IL-1 $\beta$ , or TGF $\beta$ , or IL-17A or IL-23. Stimulations were performed for 6 hours. All cytokines were used at a concentration of 100ng/ml Figure 4.21. is representative of 1 of 2 independent experiments For each independent experiment, PBMCs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point.. Statistical significance was determined using an unpaired student's t test.



**Figure 4.22. QPCR analysis of D6 transcript numbers in PBMCs stimulated with IL-17.**

PBMCs were stimulated with either PBS or IL-17A. Stimulations were performed for 2 hours, 4 hours, 8 hours or 24 hours. IL-17A was used at a concentration of 100ng/ml. Figure 4.22. is representative of 1 of 2 independent experiments For each independent experiment, PBMCs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or cytokine, per time point.. Statistical significance was determined using an unpaired student's t test.

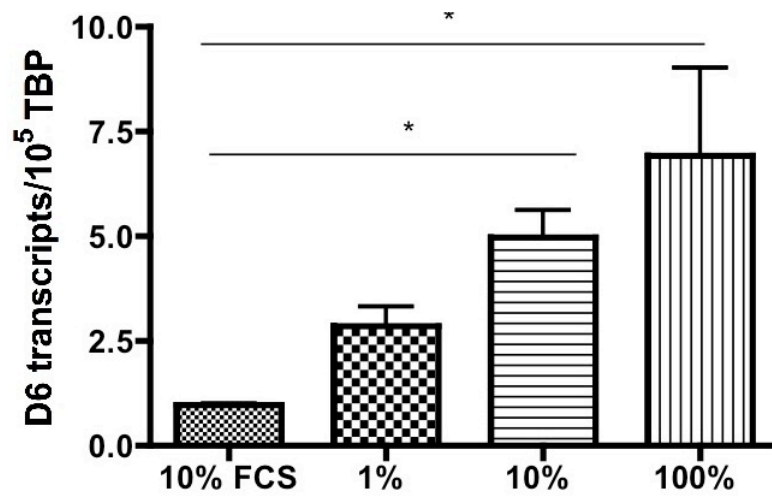


Therefore, PBMCs were stimulated again with IL-17, this time for 2 hours, 4 hours, 8 hours, or 24 hours. At each time-point, RNA was extracted from the cells, and D6 transcript numbers measured using QPCR (Figure 4.22.). As shown in figure 4.22., IL-17 (at the time points tested) did not alter the numbers of D6 transcripts in PBMCs.

#### 4.5.3. Effect of serum and plasma on PBMC-D6 expression

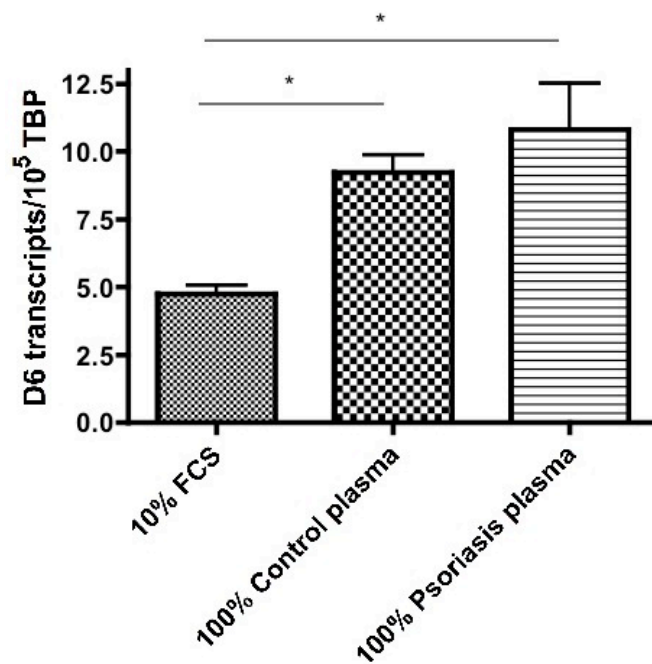
As PBMCs circulate in the blood, it is possible factors other than IFN $\gamma$  in the plasma could also contribute to the increase in the number of D6 transcripts in psoriasis PBMCs reported in Figure 3.29 and Figure 4.10. Serum was initially used to stimulate PBMCs, as serum is commercially available. However plasma from psoriasis patients was also collected over a number of months from the 'out-patient' clinics. As shown in figure 4.23., stimulating PBMCs with increasing concentrations of human serum resulted in an increase in the numbers of D6 transcripts compared to PBMCs cultured in normal media (i.e. RPMI containing 10% FCS).

To directly test whether a component of the plasma of psoriatic patients was responsible for the elevation of D6 expression in psoriasis PBMCs, whole plasma from psoriasis patients, and whole plasma from healthy controls were used to stimulate PBMCs. The levels of D6 expression were then analysed. The data obtained show that both healthy control and psoriasis plasma significantly elevated D6 expression compared with PBMCs cultured in standard media (Figure 4.24.) However, no difference in the number of D6 transcripts were noted when PBMCs were stimulated in psoriasis plasma compared with PBMCs stimulated with healthy control plasma (Figure 4.24.).



**Figure 4.23. QPCR analysis of D6 transcript numbers in PBMCs cultured in human serum.**

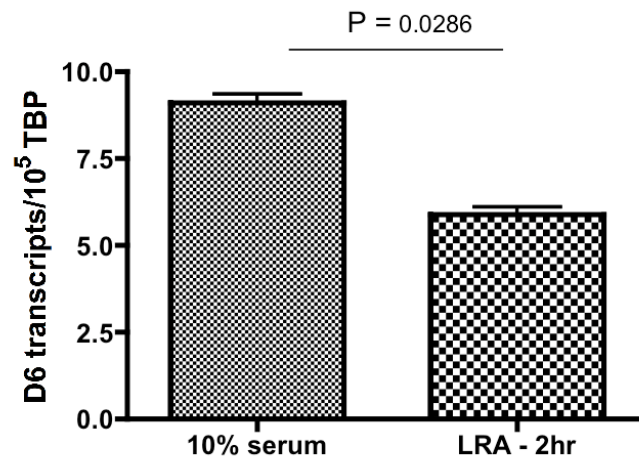
PBMCs were cultured in normal media (10% FCS), or 1% human serum, or 10% human serum or 100% human serum. Cells were stimulated for 6 hours. Statistical significance was determined using a Kruskal Wallis test and Dunn's Multiple Comparison test. Figure 4.23. is representative of 1 of 2 independent experiments For each independent experiment, PBMCs were seeded into a 24 well plate, and 4 wells were stimulated with either media, or serum concentration, per time point.



**Figure 4.24. QPCR analysis of D6 transcript numbers in PBMCs cultured in human plasma.**

PBMCs were cultured in normal media (10% FCS), or 100% healthy control plasma or 100% psoriasis plasma. Cells were stimulated for 6 hours. Statistical significance was determined using a Kruskal Wallis test and Dunn's Multiple Comparison Test. Figure 4.24. is representative of 1 of 2 independent experiments For each independent experiment, PBMCs were seeded into a 24 well plate, and 4 wells were stimulated with either PBS, or plasma, per time point.

As Figure 4.23. and Figure 4.24. show that D6 expression is elevated in PBMCs when stimulated with serum/plasma, possible mediators found in serum/plasma causing the increased D6 transcript levels were investigated. Lipids have recently been shown to be elevated in psoriasis blood compared with healthy control blood (Tekin et al., 2007). With this in mind, lipids were removed from serum using Lipid Removal Agent (LRA). The lipid depleted serum was then used to stimulate PBMCs, and compared with lipid positive serum. As shown in Figure 4.25. use of LRA to remove the lipids from serum resulted in a reduction in the numbers of D6 transcripts compared with PBMCs stimulated with serum without prior incubation with LRA. This suggests that a lipid component of serum can increase PBMC D6 transcript numbers. Collectively, these data show that PBMC-D6 expression can be regulated by various factors. The ability of IFN $\gamma$  to increase PBMC-D6 expression is of great interest when it is considered that IFN $\gamma$  can also induce notable increases in D6 expression on NHEKs and HDLECs.



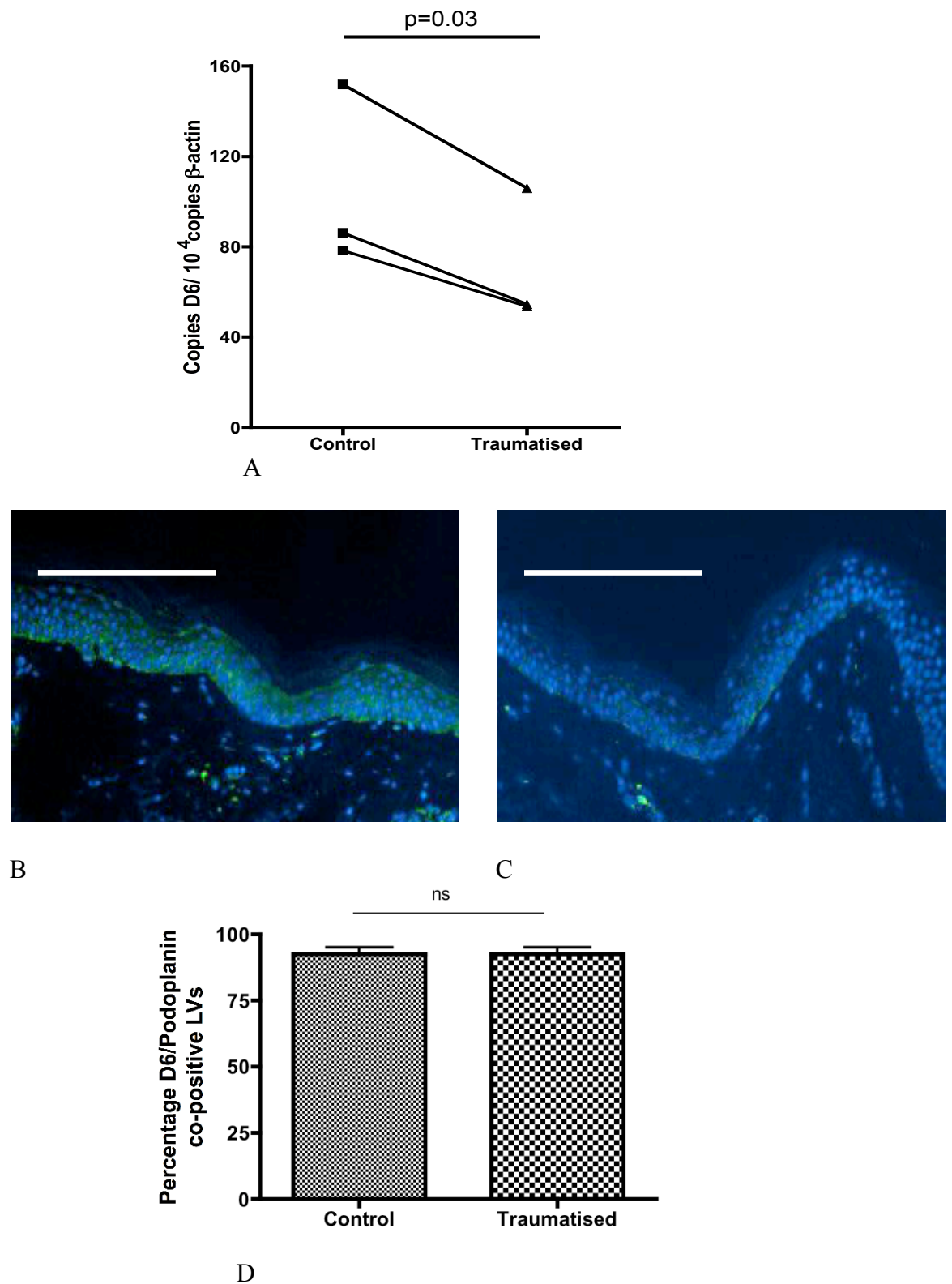
**Figure 4.25. Effect of LRA D6 expression in serum stimulated PBMCs.**

PBMCs were cultured in normal media containing 10% human serum (10% serum), or 10% serum incubated beforehand with LRA for 2hrs (LRA – 2hr). Cells were stimulated for 6 hours. Statistical significance was determined using a Mann Whitney U test. Figure 4.25. is representative of 1 of 2 independent experiments. For each independent experiment, PBMCs were seeded into a 24 well plate, and 4 wells were stimulated with either serum, or LRA, per time point.

#### **4.6. Effect of micro-trauma on D6 expression in human skin**

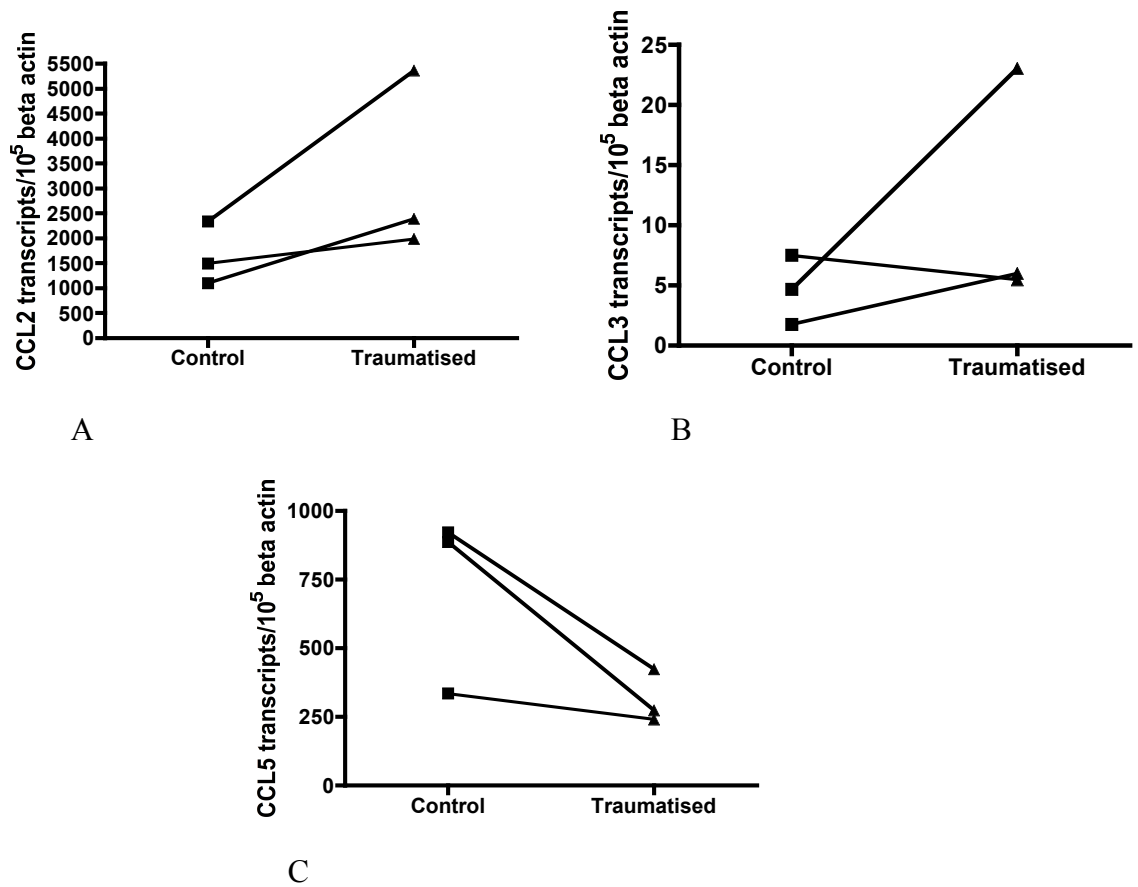
As described earlier, there is a notable reduction in D6 expression both at transcript level (Figure 3.2.), and protein level (Figures 3.9. – 3.12., 3.20., 3.22.), when comparing uninvolved psoriatic skin and peri-lesional psoriatic skin. Therefore there must be a trigger(s) which results in a transient reduction in D6 expression at this site. Psoriasis patients have a propensity to develop lesions as a result of Koebnerisation (Weiss et al., 2002), therefore it has been suggested that uninvolved psoriatic skin is ‘primed’ for lesion development. Psoriasis patients are prone to developing new lesions upon trauma to the skin (Perera et al., 2012). To test whether the high D6 levels in uninvolved psoriatic skin is reduced upon minor trauma, three psoriasis patients were subjected to 10 tape strips of an area of uninvolved psoriatic skin. The next day, biopsies were taken from the tape-stripped sites, in addition to a contralateral uninvolved site (control). D6 expression was then examined using QPCR and IF protein analysis.

As can be seen in figure 4.26.A. (thanks to Kave Shams for figure 4.26.A.), minor trauma to uninvolved psoriatic skin, resulted in a significant reduction in the numbers of D6 transcripts, compared with a contralateral non-traumatised (uninvolved psoriatic skin) control. To examine whether a reduction in D6 expression occurred in traumatised skin compared to the contralateral control, immuno-staining for D6 protein was performed. While D6 expression is found throughout the epidermis in the contralateral control (Figure 4.26.B.), D6 expression in traumatised skin is largely absent from the epidermal layer (Figure 4.26.C.). Interestingly, micro-trauma did not reduce the percentage of D6-positive LVs in traumatised (previously uninvolved) psoriatic skin (Figure 4.26.D.). These data suggest, that while micro-trauma can reduce D6 expression on the epidermis, other factors, or a combination of micro-trauma and other factors, are involved in a reduction of D6 expression on LVs. To examine whether a loss of D6 expression occurred concomitantly with elevated inflammatory CC chemokine production, QPCR for CCL2, 3 and 5 was carried-out. This proved not to be the case for CCL2, 3 or 5 as no statistical difference was observed (Figure 4.27.A., B. and C.). This result was surprising as it might be expected that a loss of D6 expression, coupled with micro-trauma, which might induce localised inflammation, would result in an increase in inflammatory CC chemokine transcript numbers. If Figure 4.27.A. is examined again, there does appear to be a slight increase in CCL2 transcript numbers. It is possible that with greater numbers of samples, a statistically significant increase in CCL2 transcripts would be observed. Collectively, these data suggest a possible mechanism by which D6 expression is reduced in peri-lesional psoriatic skin, which results in onset of lesion formation.



**Figure 4.26. Effect of micro-trauma on cutaneous D6 expression.**

A. D6 transcript numbers in control (uninvolved contralateral skin), or traumatised (previously uninvolved) psoriatic skin. IF D6 staining of B. control (uninvolved contralateral skin) or C. traumatized (previously uninvolved) psoriatic skin. D. Percentage D6/Podoplanin co-positive LVs in control uninvolved skin or traumatised psoriatic skin. Scale bars 100  $\mu$ m. Control N = 3, Traumatized N = 3. Statistical significance was determined using a paired t test.



**Figure 4.27. Effect of micro-trauma on inflammatory CC chemokine expression.**

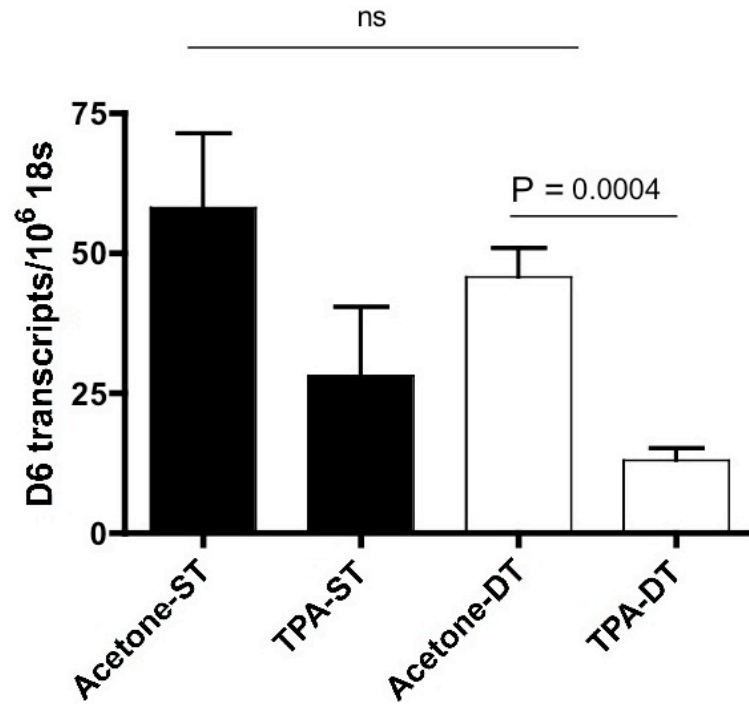
QPCR analysis of A. CCL2 transcripts B. CCL3 transcripts C. CCL5 transcripts in control (uninvolved contralateral control) and traumatized (previously uninvolved) skin from psoriasis patients. Control N = 3, Traumatized N = 3. Statistical significance was determined using a paired t test.

#### **4.7. Regulation of D6 expression using mouse models of psoriasiform pathology**

Although data described previously suggested that D6 was elevated in uninvolved psoriatic epidermal keratinocytes, as a consequence of the inflammatory cytokines found in uninvolved psoriatic skin, the exact molecular mechanism(s) behind increased D6 expression at this site, remained unknown. A recent study suggested that over-expression of the S100 proteins was responsible for this priming effect. An S100a7/a15 transgene under the control of the keratin 5 promoter was introduced into mice. The resulting mice were observed to have elevated S100a7/a15 expression throughout the epidermis (Wolf et al., 2010). In addition mice were generated expressing only S100a7 under the control of the same promoter (Wolf et al., 2010). It was noted that in S100a7/a15 transgenic mice, the skin appeared similar to that of uninvolved psoriatic skin in terms of leukocyte infiltrate and over production of antimicrobials such as LL-37 (Wolf et al., 2010). This model (and the Imiquimod model of cutaneous inflammation described later) was used to examine whether a mouse model of psoriasis-like disease, mirrored the pattern of D6 expression observed in clinical samples. If the mouse model mirrored the pattern of D6 expression in clinical samples, uninvolved skin from a mouse with psoriasis-like skin inflammation would have significantly higher D6 expression compared to the lesion. In addition, uninvolved skin in the mouse with psoriasis-like skin inflammation would also have significantly higher D6 expression compared to placebo control mouse skin. If mouse D6 expression in models of cutaneous inflammation mirrored D6 expression in clinical samples, this mouse model could be used to further study D6 in psoriasis in a relevant mouse model of the disease.

Upon irritation of the skin using TPA, the S100a7/a15 transgenic mice developed lesions on the skin similar to psoriasis (Wolf et al., 2010). To test whether D6 expression in this model mirrored the patterns of D6 expression observed in clinical samples, RNA was obtained from mice expressing S100a7 or S100a7/a15 that had been treated with TPA or acetone. As can be seen in figure 4.28. there is no difference in the level of D6 expression between acetone treated single and double transgenic mice, suggesting that S100 proteins are not essential for the elevated D6 expression in uninvolved psoriatic skin. TPA stimulated double transgenic mice resulted in a significant decrease in D6 RNA levels, whereas this significant reduction was not noted in single transgenic TPA stimulated mice (Figure 4.28.).



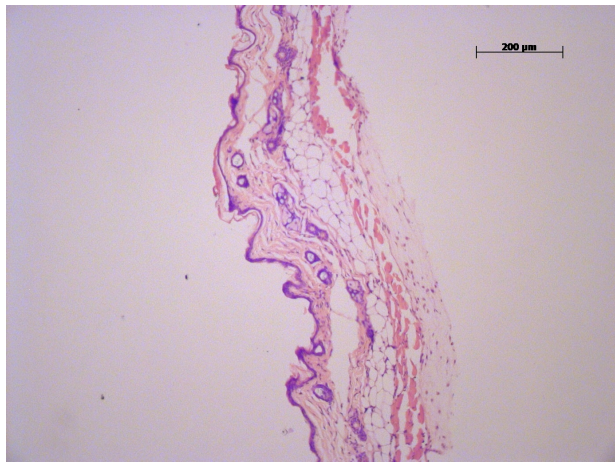


**Figure 4.28. QPCR analysis of D6 transcript numbers in acetone or TPA painted S100a7 single transgenic (ST), or S100a7/a15 double transgenic (DT) mouse skin.** Acetone painted ST N = 3, TPA painted ST N = 5, Acetone painted DT N = 3, TPA painted DT N = 5. RNA for QPCR analysis was obtained from Yuspa and colleagues (Wolf et al., 2010). Statistical significance was determined using an unpaired student's t test.

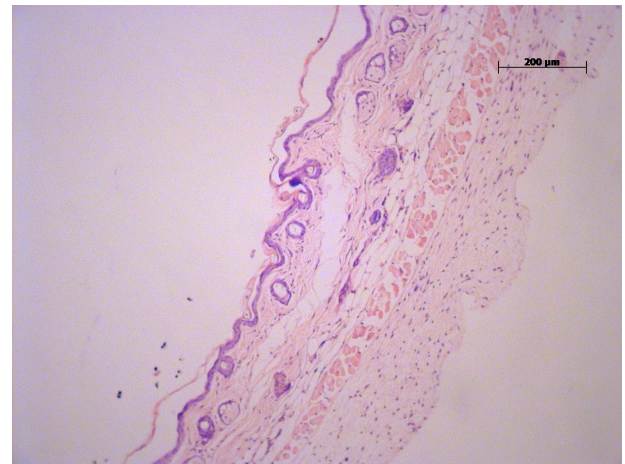
Therefore, a reduction in D6 expression was associated with a psoriasis-like pathology in this model. However, these data did not fully replicate the patterns of D6 expression observed in clinical samples. The acetone treated double transgenic mouse is similar to uninvolved psoriatic skin in psoriasis patients, as it is primed for lesion development. However, no increase in D6 expression was observed in acetone treated double transgenic mice, compared with acetone treated single transgenic mice.

The second mouse model of psoriasis-like cutaneous inflammation used to examine the increase in D6 expression at non-lesional sites was the Imiquimod mouse model (van der Fits et al., 2009). The TLR7 agonist Imiquimod was used to induce psoriasiform pathology in WT mice as described in the Materials and Methods section (Section 2.2.22). Briefly, C57BL/6 mice were shaved on the dorsal skin, and the neck. The next day, they were treated on the dorsal skin with either Imiquimod, or Vaseline control daily for 5 days. On day 6, mice were then culled, and examined for symptoms of psoriasiform pathology. As shown in Figure 4.29.A. (neck). and Figure 4.29.B. (dorsal skin), mice treated with Vaseline control did not display a psoriasiform pathology. Skin taken from Imiquimod treated mice at a site not treated with Imiquimod (neck) also did not display a psoriasiform pathology (Figure 4.29.C.). However, application of Imiquimod to the dorsal skin of the mice resulted in epidermal hyperplasia (Figure 4.29.D.), one of the defining features of psoriasiform pathology.

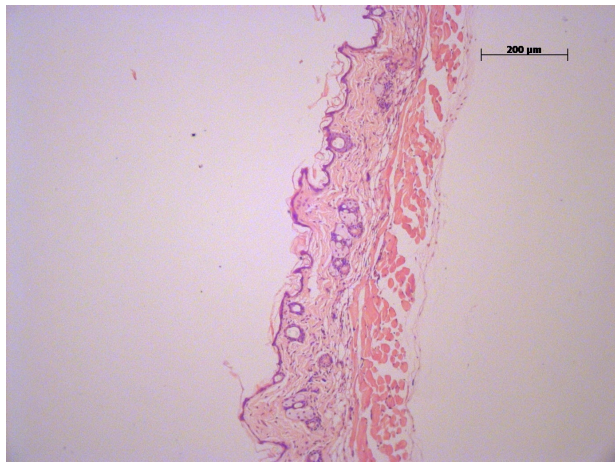
Intra-epidermal T-cell infiltration is one of the features of psoriasis (Conrad et al., 2007). Therefore, immuno-staining for CD3+T-cells was performed on Vaseline treated mouse skin, and the skin of Imiquimod treated mice. Figure 4.30.A. and Figure 4.30.B. show small numbers of T-cells in the epidermis of Vaseline treated mice. Similarly, Figure 4.30.C. show small numbers of T-cells found within the epidermis of Imiquimod treated mice at a site distant from application of Imiquimod (neck). However, Figure 4.30.D. shows multiple CD3+T-cells infiltrating the epidermis of Imiquimod treated dorsal skin. Figure 4.30.E. displays the isotype control for the CD3+T-cell stain. The intra-epidermal T-cells were counted, and displayed in figure 4.30.F. Upon examination of D6 expression in Imiquimod and Vaseline treated mice, a significant reduction in D6 expression was observed in Imiquimod treated back skin, compared with neck skin from Imiquimod treated mice (Figure 4.31.). No significant change in the levels of D6 expression was observed when comparing Imiquimod neck skin, and skin from mice treated with Vaseline, either from the neck skin, or the Vaseline treated dorsal skin. If the Imiquimod mouse model of psoriasiform pathology did mirror the expression of D6 observed in clinical



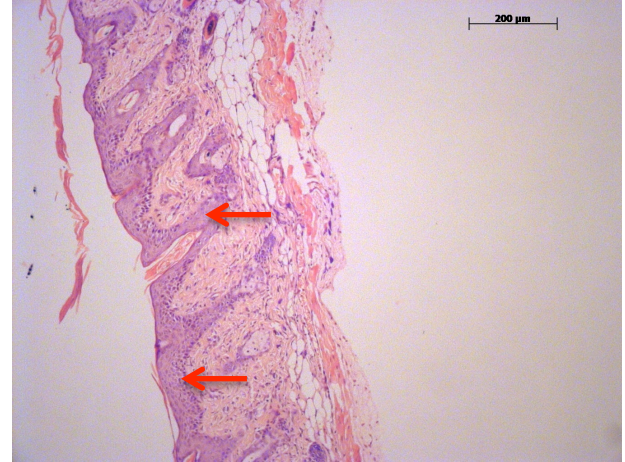
A



B



C

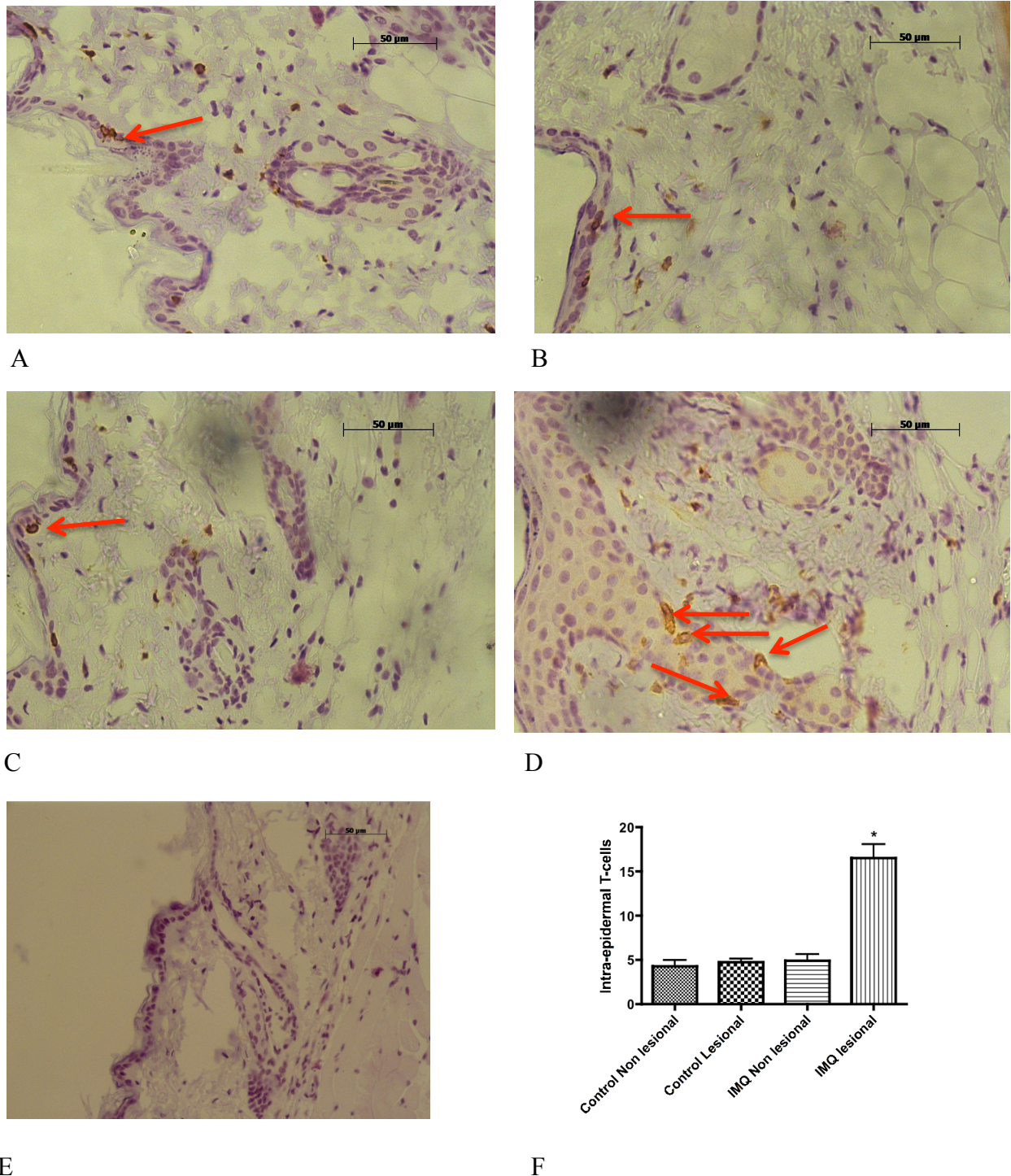


D

**Figure 4.29. Imiquimod induced psoriasiform pathology.**

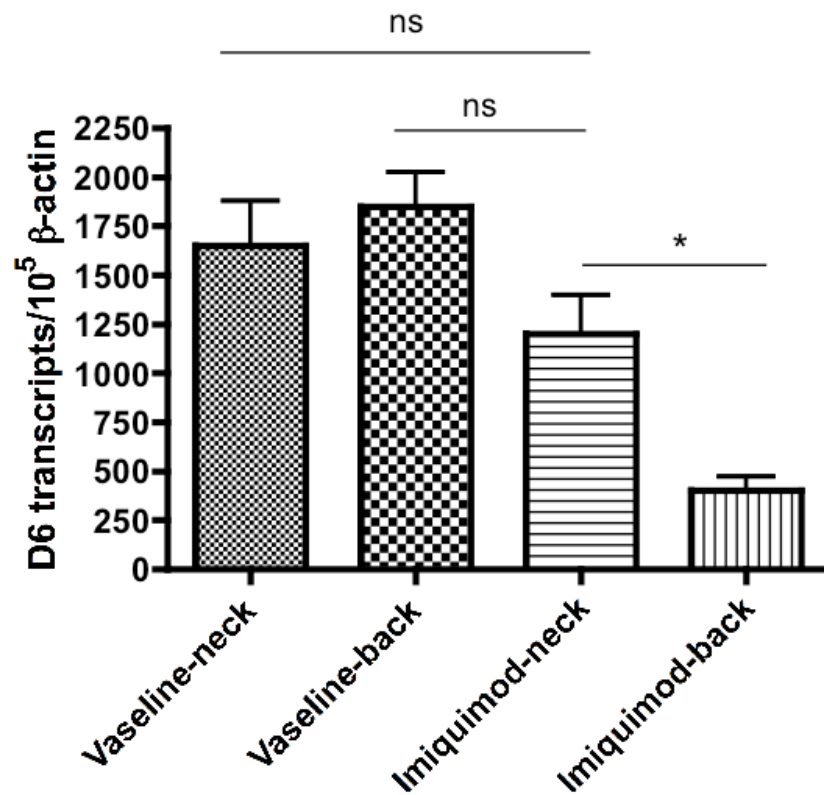
A. Neck skin from a Vaseline treated mouse B. Dorsal skin from a Vaseline treated mouse. C. Neck skin from an Imiquimod treated mouse. D. Dorsal skin from an Imiquimod treated mouse. Note the red arrow highlighting thickening of the epidermis in Imiquimod induced skin lesions. Scale bar 200μm. Eight mice were stimulated with Vaseline control or Imiquimod. The experiment was performed twice.





**Figure 4.30. Intra-epidermal T-cells in Vaseline and Imiquimod stimulated mice.**

A. Neck skin from a Vaseline treated mouse B. Dorsal skin from a Vaseline treated mouse C. Neck skin from an Imiquimod treated mouse. D. Dorsal skin from an Imiquimod treated mouse. E. Isotype control. Red arrows highlight intra-epidermal T-cells. F. Number of intra-epidermal T-cells found in Vaseline stimulated and Imiquimod stimulated. C57BL/6 mice. 4 mice were used for each stimulation. Statistical significance was determined using a one way anova test. \* $P < 0.001$ . Scale bars 50 $\mu$ m.



**Figure 4.31. QPCR analysis of D6 expression in Vaseline treated, and Imiquimod treated mice.**

Four C57BL/6 mice were used for each stimulation. Statistical significance was determined using an unpaired student's t test. \* P<0.05.

samples, Imiquimod neck skin should have significantly higher levels of D6 expression compared with Vaseline control stimulated mice. Therefore, the Imiquimod model of psoriasis-like disease confirms that a loss of D6 expression occurs when comparing non-lesional and lesional skin. However, this mouse model is not appropriate to examine the increase in D6 expression in non-lesional skin compared to Vaseline controls, as no significant increase in D6 expression was observed between these sites.

#### **4.8 Summary of chapter 4 – Regulation of D6**

As shown in chapter 3, the levels of D6 expression vary depending on the cell type, and the distance from the psoriatic lesion. The aim of this chapter was to identify and study factors that could regulate D6 expression, both *in vitro*, and *in vivo*. As various inflammatory cytokines have been previously detected in uninvolved psoriatic skin (Ohta et al., 1991, Uyemura et al., 1993, Yoshinaga et al., 1995), primary keratinocyte cells (NHEKs) and primary lymphatic endothelial cells (HDLECs) were stimulated with these, and other cytokines to test whether they could regulate D6 expression *in vitro*. As shown in this chapter, stimulation of NHEKs and HDLECs with selected inflammatory cytokines significantly increased D6 expression. Importantly, IFN $\gamma$  stimulation of NHEKs and HDLECs induced the most potent increases in D6 expression. In addition, a ‘cytomix’ of inflammatory cytokines detected in psoriatic skin (IFN $\gamma$ , TNF $\alpha$ , IL-1 $\beta$ , IL-17) was found to increase NHEK-D6 expression in a synergistic fashion.

PBMCs from a second cohort of psoriatic patients enabled an in-depth analysis of the relationship of PBMC-D6 expression with the PASI score (score used to measure disease severity), and various inflammatory mediators found in the psoriatic plasma. No correlation was observed between PBMC-D6 expression and the PASI score, therefore PBMC-D6 expression cannot be used as a prognostic marker. Luminex analysis of healthy control plasma and psoriasis plasma identified a number of inflammatory mediators that were elevated in psoriatic plasma compared to healthy control plasma. The majority of the inflammatory mediators elevated in the circulating plasma of psoriatic patients (e.g. CCL3, CCL4, CXCL8, CXCL9, IFN $\alpha$ , IFN $\gamma$ , IL-1 $\beta$ , IL-17) were not surprising as psoriasis is a Th1/17/22 cell mediated pathology, and many of these inflammatory mediators are associated with these T-cell subsets. In contrast, several Th2 cytokines (IL-4, IL-5 and IL-13), in addition to the anti-inflammatory cytokine IL-10, were also increased in the plasma of psoriasis patients compared to healthy controls. These data were unexpected as psoriasis is not considered to be a Th2 mediated pathology, and is associated with a robust inflammatory response (with reference to IL-10).

Interestingly PBMC-D6 expression positively correlated with IFN $\gamma$ , CCL4, CXCL9 and HGF. These data suggested that PBMC-D6 expression could be regulated by these factors, and stimulation of PBMCs with IFN $\gamma$  resulted in a modest, but significant increase in PBMC-D6 expression. As stimulation of NHEKs and HDLECs with IFN $\gamma$  induced striking increases in D6 expression, in addition to the increase in PBMC-D6 upon stimulation with IFN $\gamma$ , it is possible that IFN $\gamma$  is a central regulator of D6 expression. In addition to IFN $\gamma$ , PBMC-D6 expression was significantly increased by serum stimulation. Use of LRA to remove lipids from the serum resulted in a significant reduction in PBMC-D6 expression, suggesting the component of the serum responsible for increasing PBMC-D6 expression was a lipid.

The regulation of D6 expression was also examined using several *in vivo* models. It was shown that a loss of D6 expression can occur after subjecting uninvolved psoriatic skin to micro-trauma. As psoriatic lesions tend to form on the elbows, knees and scalp, which are subjected to continuous micro-trauma (Perera et al., 2012), a transient loss of D6 expression through micro-trauma may be permissible for the onset of inflammation, and subsequent lesion formation. In addition, the Imiquimod mouse model of psoriasis-like skin inflammation (van der Fits et al., 2009), and the S100a7/S100a15 double transgenic mouse model of psoriasiform pathology (Wolf et al., 2010), both showed that a loss of D6 expression occurs in the skin lesion compared to a site distant from the lesion, which is consistent with the reduction in D6 expression in lesional psoriatic skin compared to uninvolved psoriatic skin.

To conclude, in this chapter it has been shown that D6 expression can be regulated in both *in vitro* and *in vivo* contexts, relevant to the pathogenesis of psoriasis.

## **Chapter 5 – D6 in eczema**



## **5. D6 in eczema**

### **5.1. Introduction**

While psoriasis is widely considered to be a Th1/Th17/Th22 cell mediated disease, eczema is often thought of as a pathology driven by Th2 cells and cytokines (Werfel, 2009, Hanifin, 2009). There is some truth in this. Th2 cells appear to be important for the initiation of the disease, however Th1 cells are the dominant population of T-cells found in established disease as discussed previously (section 1.11.2). Therefore eczema is considered to be a biphasic T-cell mediated disease. The major symptoms of eczema are at times, intense pruritus, which causes the patient substantial discomfort and a loss of sleep. There are various allergens that can initiate eczema, including foodstuffs, in addition to inhaled substances, and infections (Werfel, 2009, Hanifin, 2009). The eczema lesions themselves contain various populations of leukocytes including T-cells (both Th2 and Th1 depending on disease progression), macrophages, eosinophils and myeloid DCs.

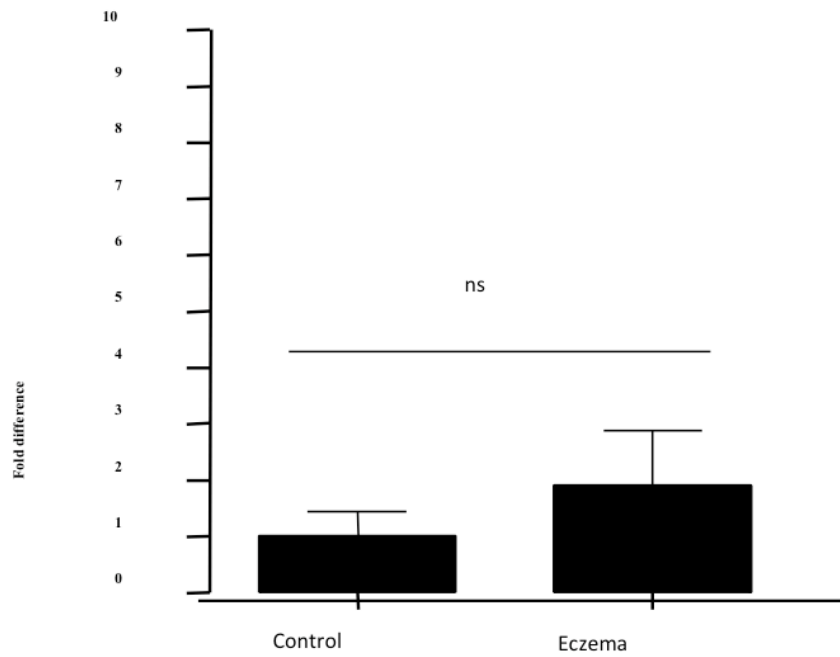
The work described in this chapter was designed to examine the expression of D6 in another cutaneous pathology distinct from psoriasis. Therefore full thickness biopsies were taken from eczema lesions, and D6 expression analysed. Specifically, the biopsies were halved, and half “snap frozen” in liquid nitrogen for later RNA extraction and purification to analyse D6 transcript numbers by QPCR. The other half was placed in formalin and paraffin-embedded for histological analysis. Similar to chapter 3, the expression of D6 on the epidermal keratinocytes and LVs in eczema sections was examined, in addition to leukocyte populations found in the dermal tissue. Finally, expression of the inflammatory CC chemokines CCL2 and CCL5 was analysed on eczematous epidermal keratinocytes.

### **5.2. Expression of D6 in healthy control and eczema skin**

QPCR analysis using RNA extracted from the biopsies shows that while there is a modest increase in the levels of D6 transcripts in eczema lesion compared to healthy controls, the difference is not significant (Figure 5.1.). Although there was no significant increase in the numbers of D6 transcripts in eczema tissue compared with healthy controls, an immunohistochemical analysis was performed to examine any subtle changes in D6 protein expression. Eczema lesions were stained for D6 using both polyclonal (Sigma), and monoclonal (R&D Systems) staining antibodies, and the immuno-reactivity analysed (Figure 5.2). No background fluorescence was detected in sections incubated with rabbit IgG fraction (Figure 5.2.A.). However, sections stained with the Sigma D6 polyclonal

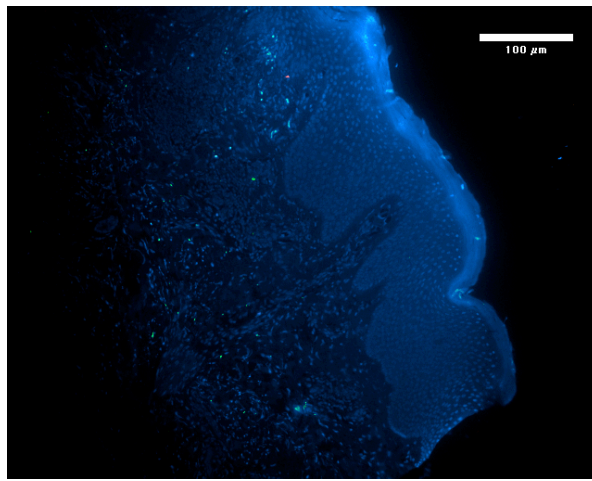
antibody resulted in detectable staining in eczema keratinocytes (Figure 5.2.B. ). Unlike psoriasis lesional staining, which is found throughout the viable epidermis (Figures 3.9. – 3.12.), D6 immuno-reactivity in eczema epidermis is predominantly found on the lower levels of the epidermis (Figure 5.2.B., highlighted with red arrows). In addition, there are some sections of the eczema epidermis that appeared to be D6 negative (See yellow arrow Figure 5.2.B.). No staining was detected in eczema sections incubated with Rat IgG2a isotype control (Figure 5.2.C.). D6 immuno-reactivity was found in eczema sections stained with the R&D Systems D6 monoclonal antibody (Figure 5.2.D.). Similar to the data in Figure 5.2.B., D6 expression in eczema lesions is generally restricted to the lower layers of the epidermis, with some layers of the epidermis lacking detectable D6 expression (highlighted by yellow arrow (Figure 5.2.D.).

As in chapter 3, D6/Podoplanin co-staining was performed (Figure 5.3.A.), and the percentage of D6/Podoplanin co-positive LVs enumerated, (Figure 5.3.B.). No increase in the percentage of D6 positive LVs was noted in eczema lesions compared with healthy controls (Figure 5.3.B.). These data are similar to data generated when examining the percentage of D6 positive LVs in psoriasis lesions (Figure 3.20.). Therefore eczema lesions have elevated D6 expression on the keratinocytes (albeit not as pronounced as psoriasis lesional keratinocytes) compared to healthy controls, similar to psoriasis lesional keratinocytes. Again similar to psoriasis lesions, there is no increase in the percentage of D6 positive LVs in eczema lesions compared to healthy controls.

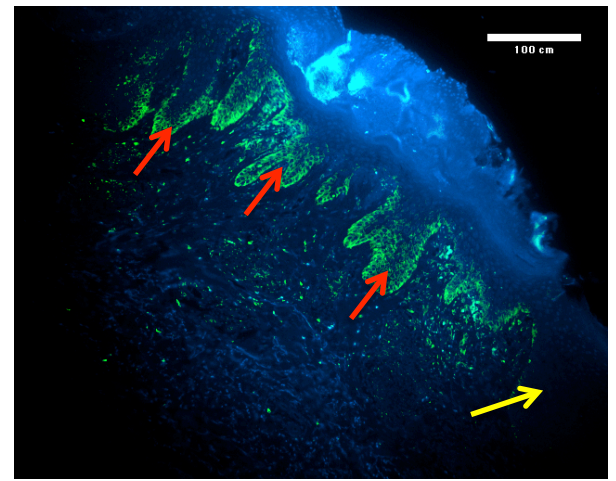


**Figure 5.1. D6 transcript numbers in healthy controls and eczema tissue.**

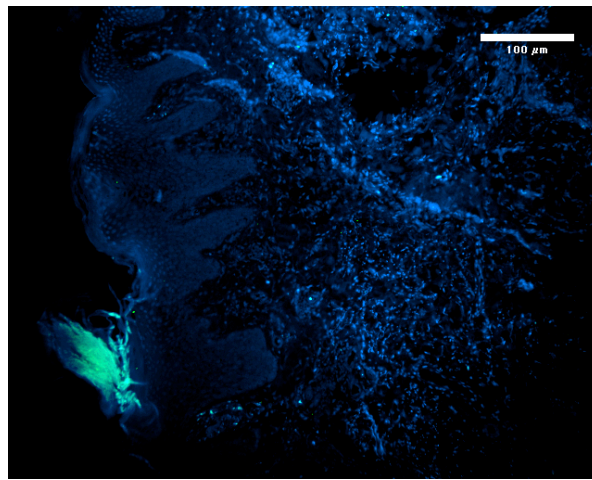
Data taken from the PhD thesis of Vicky King, 2010, “Assessment of the therapeutic potential of the atypical chemokine receptor D6”. Healthy controls N = 5, Eczema skin N = 5. Statistical significance was determined using a Mann Whitney U test.



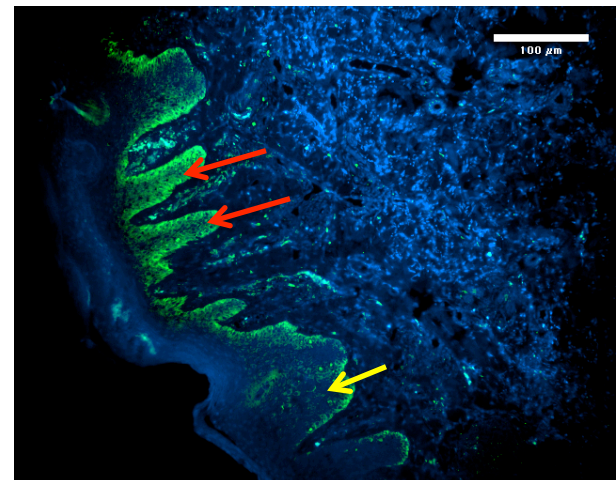
A



B



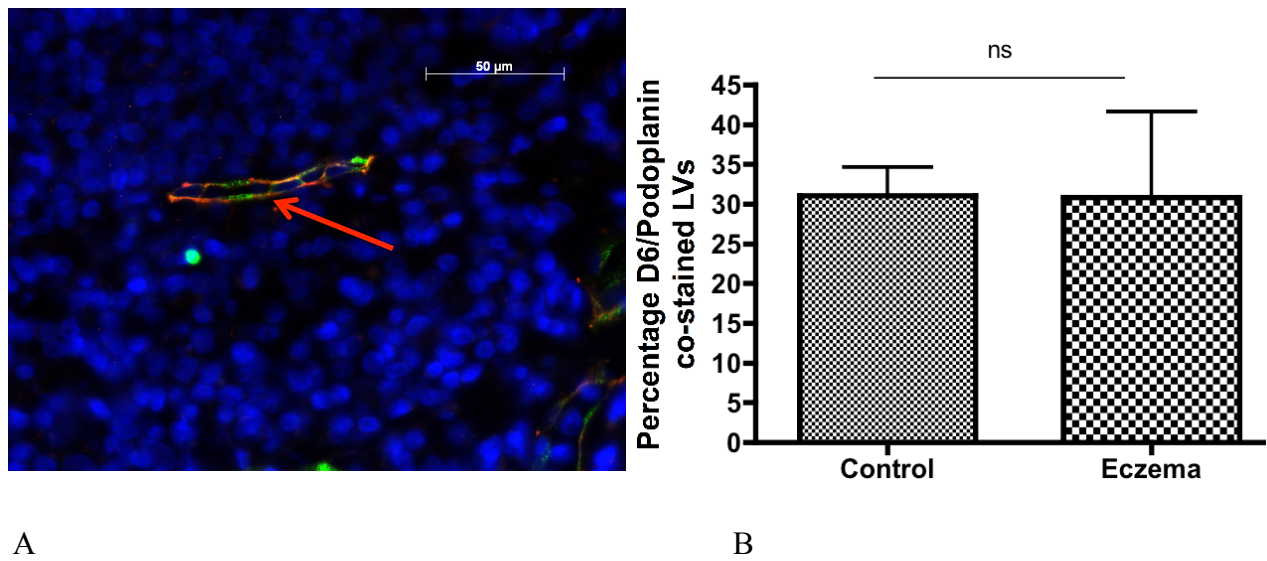
C



D

**Figure 5.2. IF D6 immuno-reactivity on eczema skin.**

A. Rabbit IgG fraction control (DAKO) incubated eczema skin sections. B. D6 (FITC) (Sigma) polyclonal immuno-reactivity detected in eczema skin sections. C. Rat IgG2a isotype control (R&D Systems) incubated eczema skin sections. D. D6 (FITC) (R&D Systems) monoclonal immuno-reactivity detected in eczema skin sections. Red arrows highlight areas of strong D6 expression. Yellow arrows highlight areas of weak D6 expression. Images are representative of immuno-stains from 5 different eczema lesions.



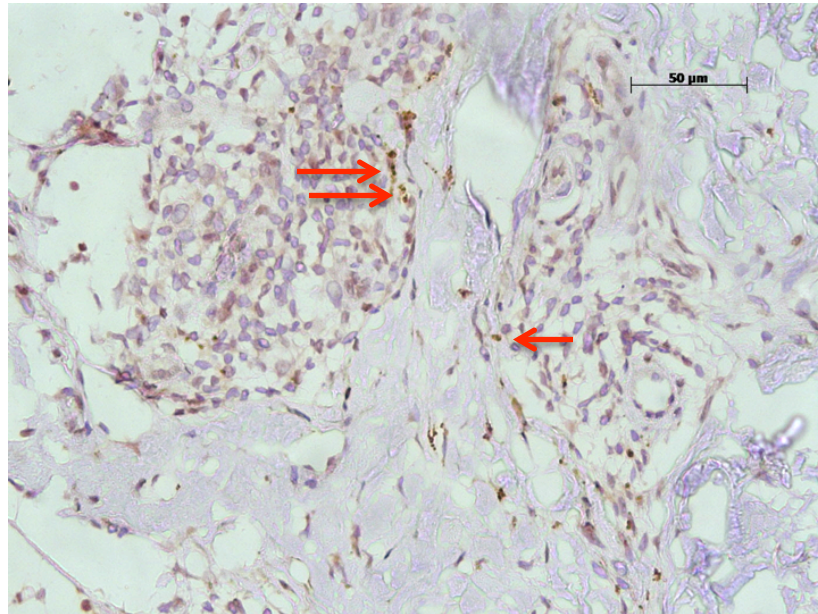
**Figure 5.3. Analysis of the expression of D6 on LVs in healthy control and eczema skin.**

A. D6 (FITC) / Podoplanin (Texas Red) co-stain. D6/Podoplanin co-positive LV (Highlighted by a red arrow). Scale bar 50μm. B. Percentage D6/Podoplanin co-positive LVs in healthy control and eczema skin. Healthy control N = 4 Eczema N = 5. Statistical significance was determined using a Mann Whitney U test.

### **5.3. D6 expression on dermal leukocytes in eczema tissue.**

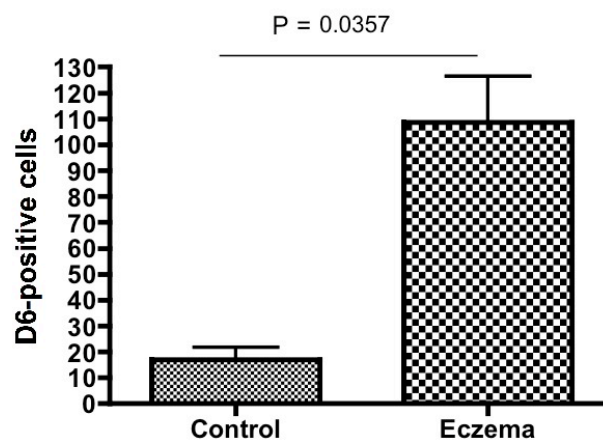
Similar to findings discussed in chapter 3, upon immuno-staining for D6, discrete populations of leukocyte-like cells also stained D6-positive in the eczema dermis (Figure 5.4.). These D6-positive cells were enumerated, and a significant increase in the numbers of D6-positive cells was observed in eczema skin compared with healthy controls (Figure 5.5.). To confirm that the D6-positive cells were leukocytes, D6/CD45 co-staining was undertaken in eczema tissue. As shown in figure 5.6 (Highlighted with red arrows), while the majority of D6 (FITC) positive cells co-stained with the leukocyte marker CD45 (Texas Red), a small number of D6-positive cells were identified that were CD45 negative (Highlighted by a white arrow, Figure 5.6.).

To phenotype the populations of D6-positive leukocytes in eczema skin, co-staining with the D6 (Sigma) polyclonal antibody and either CD68 (Macrophages), CD3 (T-cells), or Tryptase (Mast cells) was performed. D6 was visualized using FITC, and the lineage markers were visualized using Texas Red. As shown in figure 5.7., D6 positive cells were found to co-stain with CD68 (Macrophages, Figure 5.7.A.) , CD3 (T-cells, Figure 5.7.B.) and Tryptase (Mast cells, Figure 5.7.C.). No pDC stain was attempted in eczema tissue as pDCs are absent from eczema tissue, but elevated in eczema circulation (Novak et al., 2004, Wollenberg et al., 2002). The D6-positive populations were then counted, and the percentage of each D6 population calculated. The data from these counts are displayed in Figure 5.8. Approximately 87% of all D6 positive cells were accounted for when the percentage of each D6 positive population co-staining with a lineage marker were added together. Therefore, there is a population(s) of D6 positive cells that are unaccounted for. It is possible that these cells are not leukocytes as some of the D6 positive, leukocyte-like cells were also CD45 negative as shown previously (Figure 5.6.). Therefore, similar to psoriasis lesions, there are increased numbers of D6 positive leukocytes in eczema lesions.



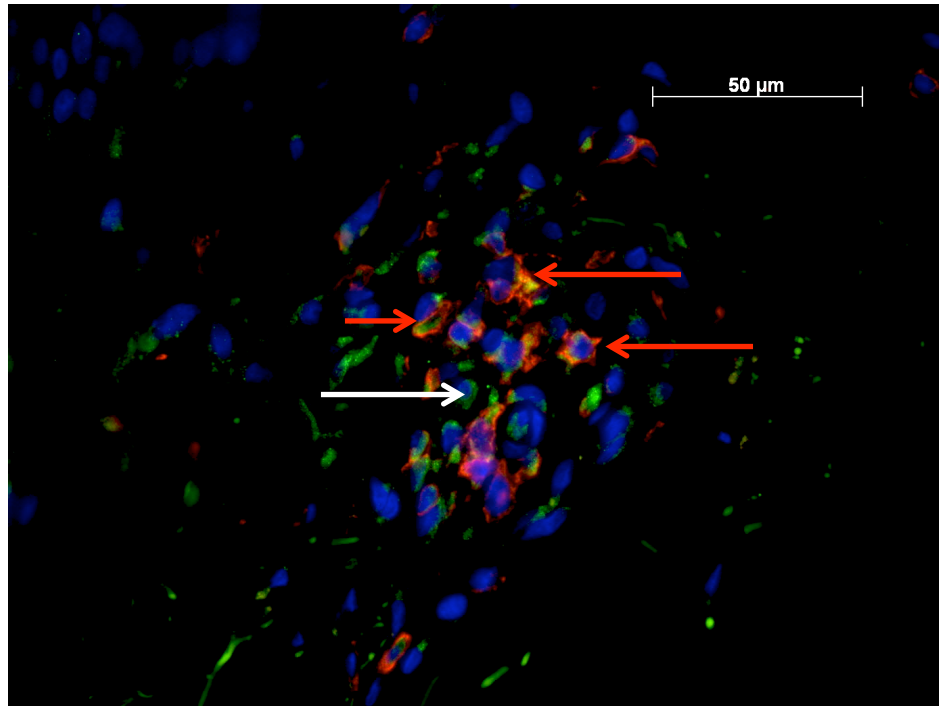
**Figure 5.4. D6 (R&D Systems) monoclonal staining of dermal cells in eczema lesions.**

Scale bar 50μm. Image representative of images taken from 5 different eczema lesions.



**Figure 5.5. Enumeration of D6-positive dermal cells in healthy controls and eczema lesions.**

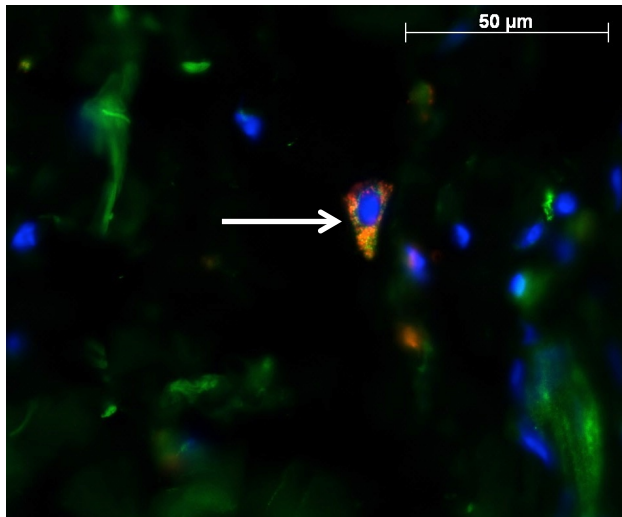
Cells were enumerated by counting D6-positive cells found in the healthy control dermal sections, and eczema dermal sections. Healthy control N = 3 Eczema N = 5. Statistical significance was determined using a Mann Whitney U test.



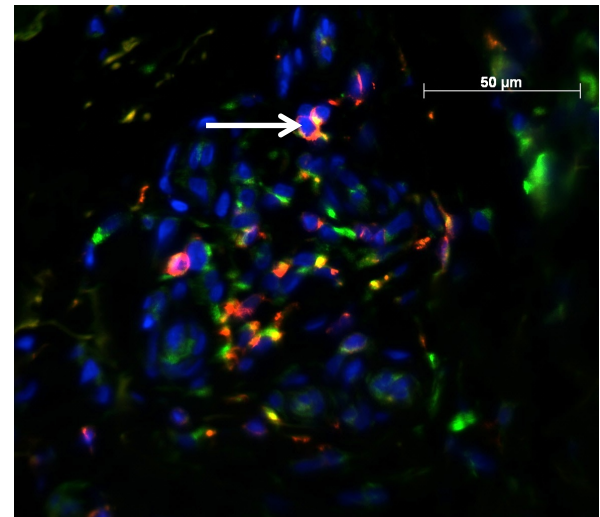
**Figure 5.6. D6/CD45 co-staining of eczema dermal cells.**

The majority of D6 positive cells (FITC) co-stain with CD45 (Texas Red) as highlighted by the red arrows. However, a discrete D6 positive, CD45 negative population, can be found as highlighted by the white arrow. Scale bar 50μm. This Image is representative of images taken from 5 different eczema lesions.

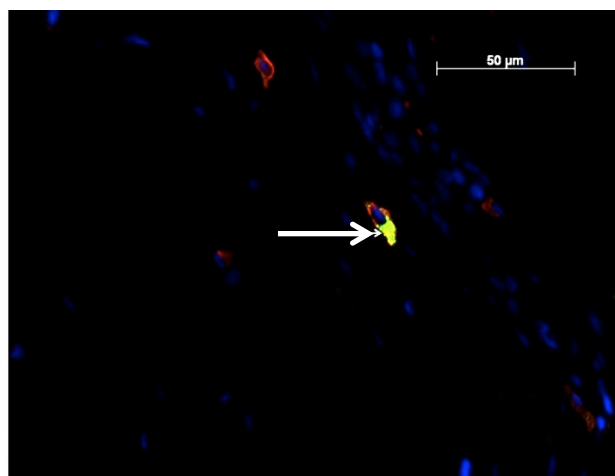




A



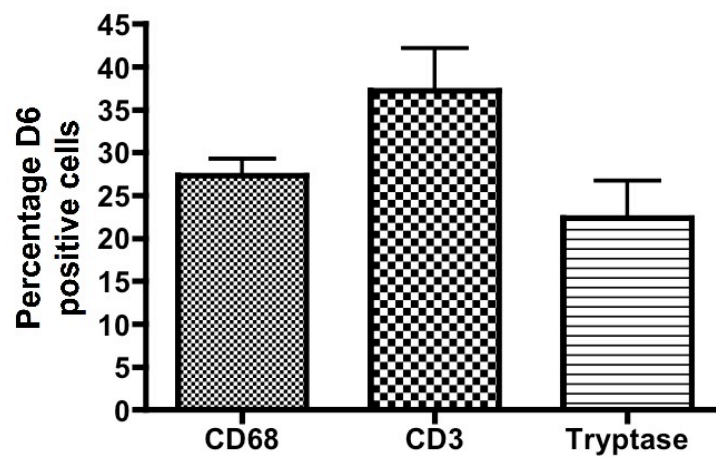
B



C

**Figure 5.7. D6 positive leukocytes co-stained with leukocyte markers in eczema lesional skin.**

Leukocytes were stained with D6 (Sigma) polyclonal antibody (FITC) and antibodies to A. CD68 B. CD3, C. Tryptase. All leukocyte markers were detected using Texas Red as the fluorochrome. Scale bars A - C - 50μm. D6 co-stained leukocytes are highlighted with white arrows. Images are representative of 5 different eczema lesional sections tested.



**Figure 5.8. Percentage D6 positive leukocyte populations in eczema skin.**

The above data were generated by counting all D6 positive cells in multiple fields of view (positive and negative for leukocyte co-stain). The data were then converted into a percentage, and displayed above. 5 different eczema samples were stained for each of the above co-stains.

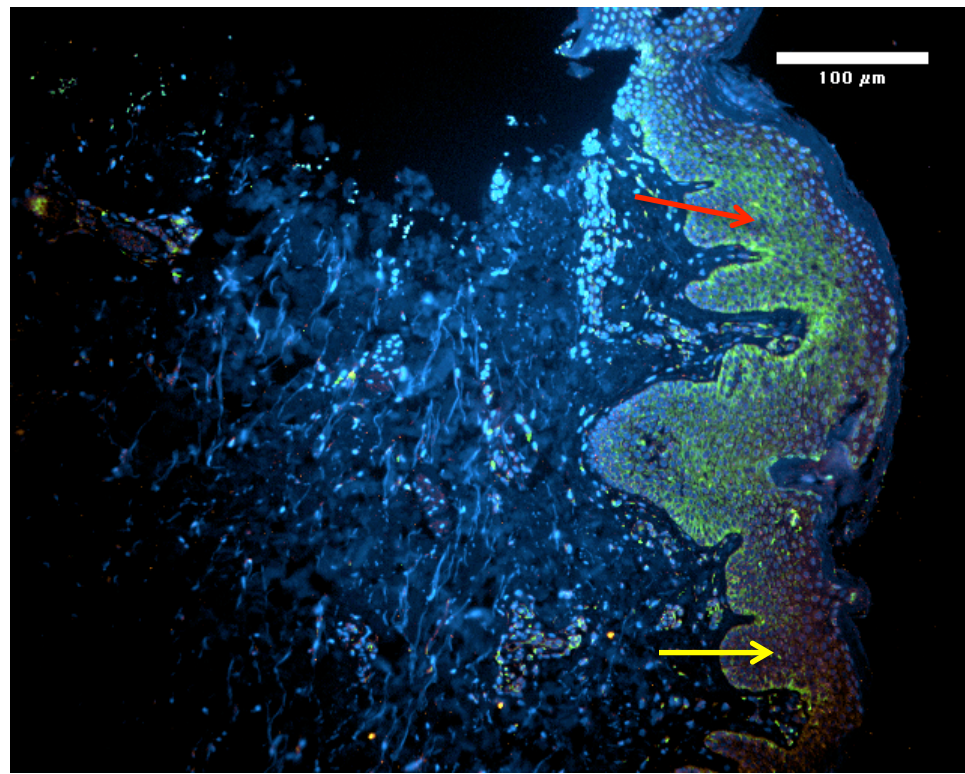
#### **5.4. Expression of inflammatory CC chemokines in eczema.**

As shown in previous studies, CCL5 is produced by the keratinocytes in eczema skin (Rebane et al., 2012). If figure 5.2. is examined closely it is clear that immuno-reactivity of D6 in the epidermal keratinocytes of eczema lesions is quite varied. Specifically, there are areas of the eczema tissue that stain positive for D6 from the lower levels up to the mid-levels of the epidermis (Figure 5.2.B. and D., Highlighted with red arrows). There are also sections of the epidermis that are essentially D6 negative (Figure 5.2.B. and D., highlighted with yellow arrows). To examine the relationship, if any, that inflammatory CC chemokines have with D6 in eczema keratinocytes, a co-stain was developed using the D6 (Sigma) polyclonal antibody, and an anti-CCL5 antibody (Figure 5.9.A.). Interestingly, the sections of the epidermis that are essentially D6 negative (Figure 5.9.A., yellow arrow), show detectable CCL5 immuno-reactivity throughout this area. There are other sections that are strongly D6 positive, but CCL5 immuno-reactivity has very weak expression (Figure 5.9.A., red arrow). Eczema tissue incubated with rabbit and goat IgG fractions had no detectable immuno-reactivity (Figure 5.9.B.).

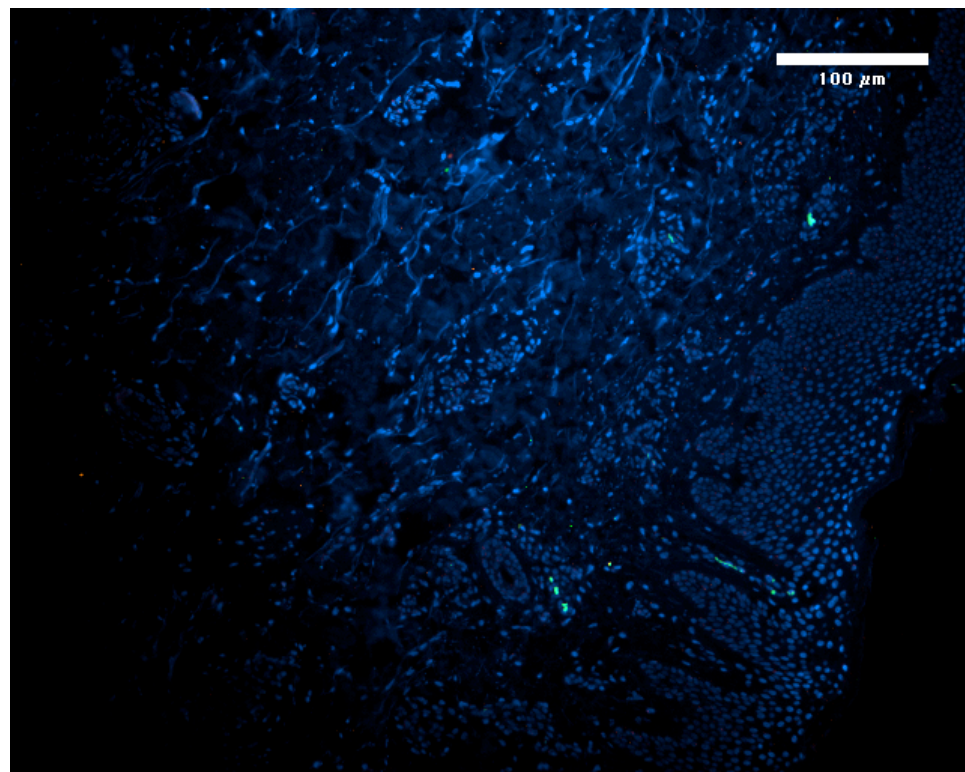
Interestingly, a co-stain of eczema lesions for D6 and CCL2 showed a similar pattern of D6 to inflammatory CC chemokine distribution. Specifically, areas of D6 expression covering the lower to mid-levels of the epidermis were noted (Figure 5.10.A. by red arrow). Concomitantly, there were sections of lesional eczema epidermis, predominantly towards the upper layers of the epidermis, that were negative for D6, but had detectable CCL2 expression (highlighted by yellow arrow). Eczema sections incubated with Rabbit IgG fractions and mouse isotype control had no detectable immuno-reactivity (Figure 5.10.B.)

The co-stains for D6 and CCL2 and CCL5 show a distribution of inflammatory CC chemokine, and D6, similar to that observed in psoriasis. Specifically, in psoriasis lesions, D6, and CCL2 and CCL5 are found throughout the epidermis. However, the strongest D6 expression is found towards the lower layers of the psoriasis epidermis, whereas the strongest layers of CCL2 and CCL5 is found towards the upper layers of the psoriasis epidermis. This is clearly displayed by the D6/CCL2 co-staining discussed earlier. A similar pattern is observed in eczema lesions. Specifically, D6 expression is focused predominantly towards the lower layers of the epidermis, with CCL2 and CCL5 found predominantly towards the upper layers of the epidermis. However, in eczema, there are sections of the epidermis that are essentially D6 negative, with strong CCL5 staining found

throughout the viable epidermis. Therefore, the D6/CC chemokine staining pattern in epidermal keratinocytes is similar in psoriasis and eczema, but not identical.



A

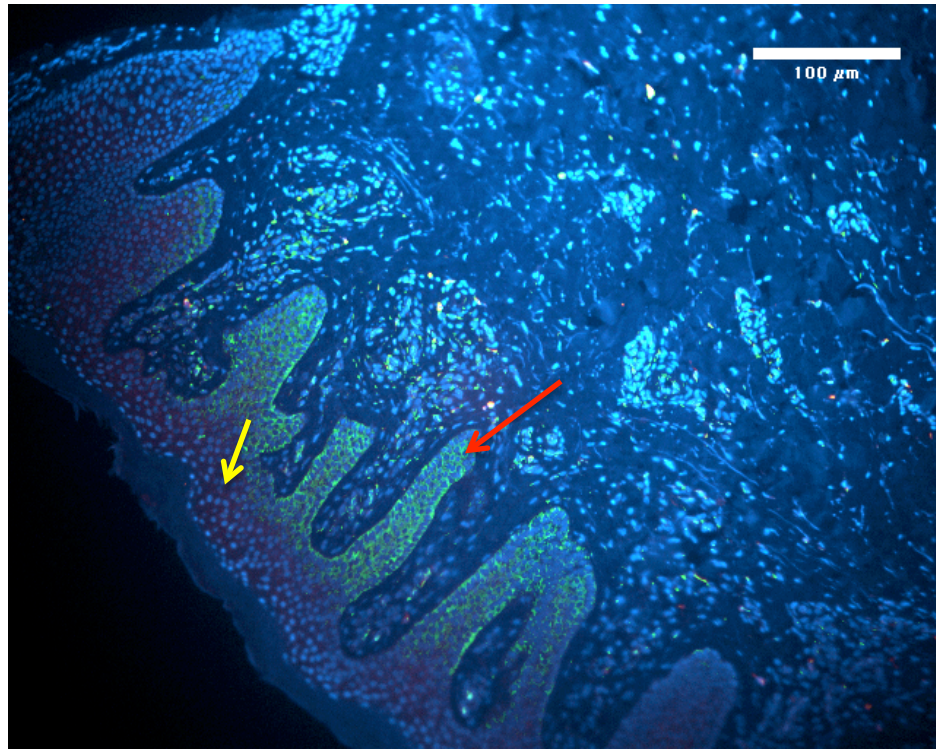


B

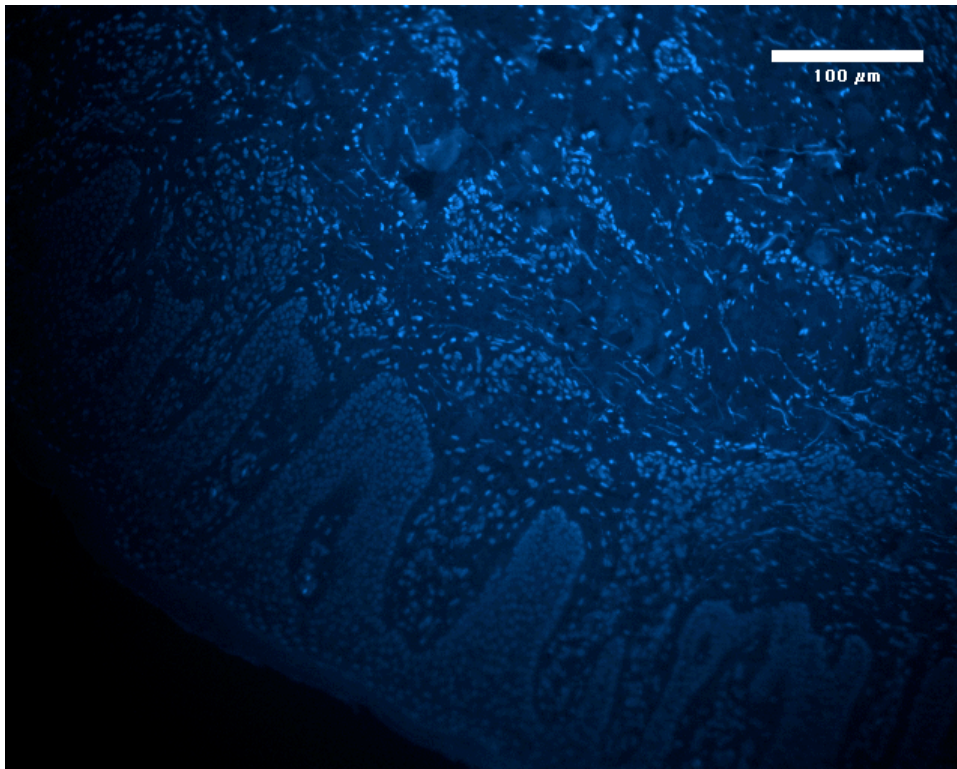
**Figure 5.9. D6/CCL5 co-staining in eczema epidermis.**

A. D6 (Sigma) polyclonal (FITC) and CCL5 (Texas Red) staining of eczema epidermis. The red arrow highlights strong D6 staining, the yellow arrow highlights CCL5 staining. B. Eczema section incubated with Rabbit IgG fraction and Goat IgG fraction. Images are representative of 4 different eczema lesional sections tested. Scale bar - 100μm.





A



B

**Figure 5.10. D6/CCL2 co-staining in eczema epidermis.**

A. D6 (Sigma) polyclonal (FITC) and CCL2 (Texas Red) staining of eczema epidermis. The red arrow highlights strong D6 staining, yellow arrow highlights CCL2 staining. B. Eczema section incubated with Rabbit IgG fraction and Mouse isotype control. Images are representative of 4 different eczema lesional sections tested. Scale bar 100μm.

### **5.5. Summary of chapter 5 – D6 in eczema**

As shown in chapter 3, D6 expression was significantly elevated in psoriatic skin compared to healthy controls. The aim of this chapter was to study D6 expression in another cutaneous pathology to examine whether elevated D6 expression, particularly on the epidermal keratinocytes, was exclusive to psoriasis, or was a systemic response to skin inflammation. No significant increase in D6 RNA expression was observed in eczema lesions compared to healthy controls. More D6 immuno-staining was detected on the epidermis of eczema lesions compared to healthy controls. D6 expression in eczema lesions localised towards the lower layers of the epidermis, in contrast to psoriatic lesions, where D6 immuno-reactivity was found throughout the viable epidermis.

Similar to psoriatic lesions, no difference in the percentage of D6 positive LVs was observed in eczema lesions compared to healthy control skin. In addition, the number of D6 positive leukocytes in eczema lesions was observed, again similar to psoriasis. Finally, the distribution of inflammatory CC chemokines and D6 on the epidermis in eczema lesions in some ways mirrored the immuno-staining observed in psoriatic lesions. Specifically, the inflammatory CC chemokines were found predominantly in the upper layers of the epidermis, whereas D6 expression was found on the lower layers of the epidermis. Therefore, D6 on the epidermis appears to be forming a barrier, possibly to prevent the release of inflammatory CC chemokines into the dermis.

To conclude, in eczema lesions, similar to psoriatic lesions, an increase in D6 protein expression compared to healthy controls is observed. However the increase in D6 expression is far subtler in eczema than in psoriasis. In addition, we have data which suggest D6 expression is increased in a third cutaneous disease SSc, compared to healthy controls (Codullo V, Singh MD et al, manuscript under revision). Collectively, these data suggest that increased D6 expression may be a feature of cutaneous disease.

## **Chapter 6 – General Discussion**



## 6.1. Discussion – Summary of work

During this project, we analysed the levels of D6 expression in psoriasis and eczema samples, compared to healthy controls. In psoriatic skin, D6 expression was significantly increased compared to healthy control skin. In particular, at sites distant from the psoriatic lesion, i.e. uninvolved psoriatic skin, D6 expression was strikingly elevated. An analysis of uninvolved psoriatic skin identified inflammatory CC chemokine expression in the epidermis, which was apparently unable to mediate the recruitment of leukocytes, as reflected by the absence of lesions at this site. In addition, D6 expression in circulating PBMCs from psoriasis patients was significantly increased in relation to healthy control PBMCs. These data lead us to suggest that D6 is elevated at sites not involved in the pathology in an attempt to ‘dampen down’ the inflammatory CC chemokine driven immune response from taking hold, and the onset of lesion formation.

To identify which mediator(s) were responsible for increasing D6 expression in uninvolved psoriatic skin, primary human keratinocytes and lymphatic endothelial cells were stimulated with inflammatory cytokines known to be expressed in uninvolved psoriatic skin (Lowes et al., 2008, Ohta et al., 1991, Uyemura et al., 1993, Yoshinaga et al., 1995). In this study we show that D6 expression could be significantly increased *in vitro* upon stimulation of human primary keratinocytes or lymphatic endothelial cells with selected inflammatory cytokines detected in uninvolved psoriatic skin. In addition, we observed a reduction in D6 expression when comparing uninvolved psoriatic skin and peri-lesional psoriatic skin. Micro-trauma to uninvolved psoriatic skin was shown in this study to result in a reduction in D6 expression. Collectively, these data suggested that a loss of D6 expression, possibly mediated by micro-trauma, was associated with lesion formation.

In eczema, D6 protein expression was elevated compared to healthy controls, but less so compared with psoriasis. There was an increase in the number of D6 positive leukocytes in the eczema dermis, and an increase in epidermal D6 expression in eczema lesions, compared to healthy controls. In addition, inflammatory CC chemokine expression was detected in eczema lesions, similar to psoriatic lesions.

### **6.1.1. Discussion of chapter 3 – D6 in psoriasis**

As discussed in the introduction, deletion of D6 in mice, coupled with treatment of the shaved dorsal mouse skin with phorbol ester, resulted in an exaggerated inflammatory pathology which resembled the human disease psoriasis (Jamieson et al., 2005). To examine whether a loss of D6 expression was associated with lesion formation in psoriasis, clinical samples were analysed.

#### **6.1.1.1. D6 RNA expression in psoriasis**

Analysis of D6 RNA expression in psoriatic skin and circulating PBMCs, compared to healthy controls provided striking results. D6 expression was significantly higher in psoriatic skin compared to healthy control skin (Figure 3.1.). Interestingly, in uninvolved psoriatic skin, D6 expression was significantly higher compared to peri-lesional psoriatic skin and lesional psoriatic skin. In circulating PBMCs from psoriasis patients, D6 RNA expression was also significantly increased compared to healthy control PBMCs (Figure 3.29.). These data disproved our initial hypothesis,

‘a loss of D6 expression is associated with lesion formation in psoriasis’.

Thus our hypothesis was revised to the following,

‘D6 expression is reduced in psoriatic lesions, in comparison to uninvolved skin’.

#### **6.1.1.2. Epidermal D6 expression in psoriasis**

In light of the previous data, the obvious questions that came to mind were,

1. Did the increase in D6 RNA result in notable increase in D6 protein?
2. If an increase in D6 protein was observed, where did the increased D6 protein localize to?
3. How was D6 expression regulated?

To examine D6 protein expression, immuno-staining for D6 was performed. As shown in Figures 3.9.-3.12., D6 expression was detected on the epidermal keratinocytes. This was of great interest, as D6 expression had not as yet been identified on the epidermis. In uninvolved psoriatic skin, which was the site of the striking 16-fold increase in D6 RNA expression (Figure 3.1.), D6 expression was found throughout the viable epidermis. This is in contrast with healthy controls, where epidermal D6 expression was restricted to the lowest level of the epidermis, the stratum basale. Therefore the increase in D6 RNA expression in uninvolved psoriatic skin was mirrored by an increase in D6 protein, localizing to the epidermis.

The next obvious question is why is D6 expression found throughout the viable layers of the epidermis in uninvolved psoriatic skin? As uninvolved psoriatic skin is histologically normal, why a chemokine-scavenging receptor would be so markedly elevated at this location was of great interest. One possibility was that D6 was elevated in uninvolved psoriatic skin in an attempt to block an inflammatory CC chemokine driven immune response from taking hold, which could otherwise result in lesion formation. To test this, immuno-staining for CCL2 and CCL5, both high affinity D6 binding ligands (Graham, 2009) was performed on healthy control skin and psoriatic skin. In agreement with other findings (Fukuoka et al., 1998, Giustizieri et al., 2002, Giustizieri et al., 2001, Raychaudhuri et al., 1999), CCL2 and CCL5 were not detected in healthy control skin. However, in uninvolved psoriatic skin, CCL2 and CCL5 were detected on the epidermis (Figure 3.15.-3.16). As uninvolved psoriatic skin is free of lesions, this would suggest that CCL2 and CCL5 in the uninvolved psoriatic epidermis are unable to direct the migration of cells into the skin, possibly due to neutralisation by D6 in the epidermal keratinocytes.

However, although uninvolved psoriatic skin has no histological abnormalities, there have been reports of increased leukocyte populations in uninvolved skin compared to healthy control skin, including NK cells and CD4<sup>+</sup> cells (Cameron et al., 2002, Yao et al., 2011). Therefore, the leukocytes must be migrating into uninvolved psoriatic skin via some sort of migratory signal. How then do leukocytes migrate into uninvolved psoriatic skin? It is possible that some of the epidermal-derived inflammatory CC chemokines escape degradation by epidermal-D6, and are released into the dermis. A review of the literature finds very little information on the expression of inflammatory chemokines in uninvolved psoriatic skin. There are some data suggesting that CXCL8 is expressed in uninvolved psoriatic skin (Jiang et al., 2001), and as NK cells express a variety of CXC and CC chemokine receptors, including CXCR1 (which binds to CXCL8) (Berahovich et al., 2006), this could result in the influx of NK cells into uninvolved psoriatic skin (Cameron et al., 2002). However, as CXCL8 is most often associated with the migration of neutrophils to the site of inflammation (Rot and von Andrian, 2004), one might expect to find neutrophils in uninvolved psoriatic skin, but these cells are not found at this site. At present these observations cannot be reconciled. It is possible that other inflammatory chemokines are expressed in uninvolved psoriatic skin, (as only CCL2 and CCL5 were examined in this study) which may account for the recruitment of leukocytes into uninvolved psoriatic skin. Alternatively it is possible dermal cells could produce inflammatory chemokines, allowing the migration of leukocytes into uninvolved psoriatic skin.

There is a notable reduction in D6 protein expression on the epidermis of peri-lesional psoriatic skin compared to uninvolved psoriatic skin (Figures 3.9.-3.12.). A similar reduction in D6 expression is observed when comparing the levels of D6 RNA in uninvolved and peri-lesional psoriatic skin (Figure 3.1.). Therefore, directly adjacent to the lesion, there is a significant drop in D6 expression. These data are of interest when it is considered that in addition to expression in uninvolved psoriatic skin, CCL2 and CCL5 are detected in the epidermis of peri-lesional and lesional psoriatic skin (Figures 3.15. and 3.16.). The reduction of D6 expression observed in peri-lesional psoriatic skin could result in a release of CCL2 and CCL5 into the dermal regions, which could result in the onset of lesion formation.

D6 expression in lesional psoriatic epidermis, similar to uninvolved psoriatic epidermis is found throughout the viable layers. The next question to be asked is if D6 is found throughout the viable epidermis in lesional psoriatic skin, why then does lesion formation still occur? From this study, this question cannot be answered. However it is possible the reduction of D6 expression allows a new lesion to start forming, and once the lesion is established, the ability of epidermal-D6 to degrade inflammatory CC chemokines produced by the epidermis may be overwhelmed. While this study focused on the role of inflammatory CC chemokines produced in the epidermis, it is possible that inflammatory chemokines produced by the dermis could explain why lesion formation occurs, despite D6 expression throughout the viable epidermis. Therefore, it is possible that D6 on the epidermis in psoriatic lesions is successfully blocking the release of inflammatory CC chemokines into the dermis. The D6/CCL2 co-stain (Figure 3.17.) clearly shows that while both D6 and CCL2 are found throughout the epidermis, D6 expression is strongest towards the lower layers of the epidermis, whereas CCL2 expression is strongest towards the upper layers of the epidermis. Collectively, these data suggest a role for epidermal-D6 as a barrier to unrestricted inflammatory CC chemokine release into the tissue. As the epidermal keratinocytes are a major site of chemokine production (Mabuchi et al., 2012), D6 on the epidermis is ideally located to prevent unwanted inflammatory CC chemokine release into the dermis.

As shown in Figure 3.1., the levels of D6 RNA in peri-lesional and lesional psoriatic skin are the same, yet there is a considerable increase in D6 protein on the epidermis of lesional psoriatic skin (Figures 3.9.-3.12.). Why then is there no difference in the level of D6 RNA between peri-lesional and lesional psoriatic skin, yet there is such a marked difference in D6 protein? Quite simply, these data cannot be fully explained on the basis of this study.

However, it is possible that the increase in D6 protein is due to a localised increase in D6 expression on the epidermis, and the ‘base-line’ D6 RNA expression from the full thickness biopsies is being set by another cell type (possibly the LVs). LCM was used to test whether an increase in D6 RNA expression between peri-lesional and lesional psoriatic epidermis occurred. However, while there was a general increase in D6 RNA levels in lesional psoriatic epidermis compared to peri-lesional psoriatic epidermis, this did not reach statistical significance (Figure 3.14.). Although it has been successfully performed previously (Specht et al., 2001), formalin fixed tissue is not ideal for LCM, as formalin fixation can result in degradation of the RNA. A more reliable way of testing whether the increase in D6 protein in the lesional psoriatic epidermis is mirrored by an increase in D6 RNA would be to obtain new biopsies, and then freeze them. This freezing process would protect the RNA from degradation. Frozen sections could then be cut from these biopsies, and LCM analysis performed as before. This would ensure more reliable RNA quality, and subsequently improve the reliability of results.

#### **6.1.1.3. LV D6 in psoriasis**

D6 expression was found throughout the viable epidermis of uninvolved and lesional psoriatic skin, yet there was a significant reduction in D6 RNA expression in lesional psoriatic skin compared to uninvolved psoriatic skin. Therefore it is unlikely that epidermal-D6 was solely responsible for the approximate 16-fold increase in D6 RNA expression in uninvolved psoriatic skin. Therefore, other more established D6-positive cells were analysed for D6 expression. The LVs are the most studied site of D6 expression (McKimmie et al., 2013, Nibbs et al., 2001). Co-staining for D6, and the lymphatic endothelial cell marker podoplanin (Breiteneder-Geleff et al., 1999), showed that a subset of LVs were D6 positive, confirming previous results (Nibbs et al., 2001). Interestingly, D6 expression on LVs was biased towards the lumen of the LV (Figure 3.19.A.). However, D6 expression was also detected (albeit weakly) on the sub-luminal surfaces of the LV, as evidenced by the co-staining, which resulted in an ‘orange-hue’ on the sub-luminal side of the LV (Figure 3.19.A.). These data suggest that D6 on LVs can bind and internalize inflammatory CC chemokines on both luminal and subluminal LV surfaces. In inflamed D6 KO mice, inflammatory leukocytes were found to bind to the luminal and sub-luminal lymphatic surfaces, due to the accumulation of inflammatory CC chemokines at this site (Lee et al., 2011). Therefore, D6 expression on both the luminal, and sub-luminal sides of the LVs is consistent with previous observations by Graham and colleagues (Lee et al., 2011). As shown in Figures 3.20. and 3.22., almost all LVs (95%) were D6 positive in uninvolved psoriatic skin. In contrast, only approximately 25% of all LVs in healthy

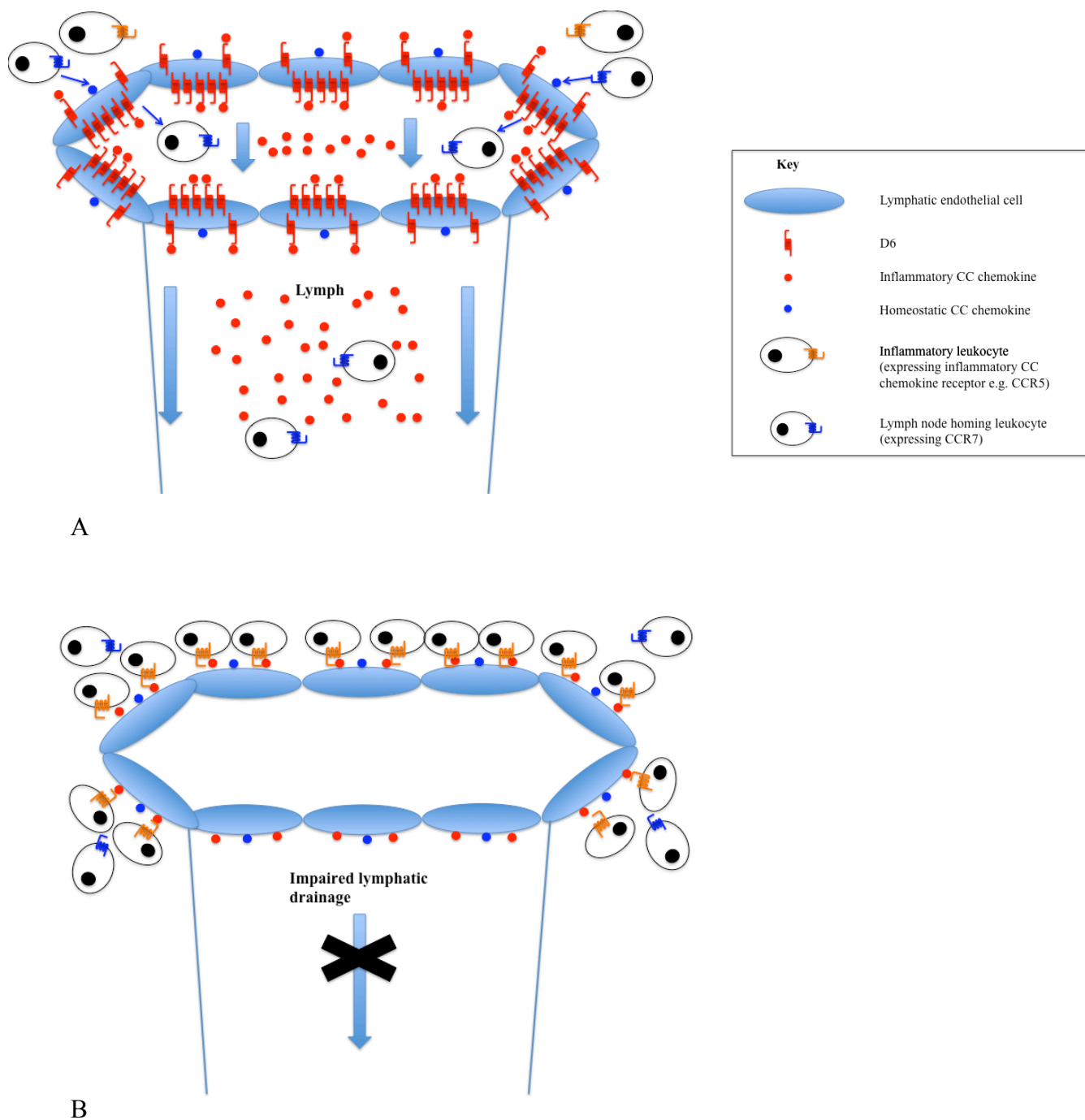
control skin and peri-lesional and lesional psoriatic skin were D6 positive. Therefore in uninvolved psoriatic skin, all of the viable epidermis, in addition to almost all of the LVs are D6 positive. These data suggest that LV-D6 in uninvolved psoriatic skin contribute's significantly to the increase in D6 transcripts at this site. It must be conceded that a limitation of these data was the data's subjectivity. The author performed both sets of immuno-staining (D6 serial sections and D6/Podoplanin co-staining), and while extreme efforts were made on the part of the author to avoid biasing the data, this cannot be conclusively discounted. If the author were to perform these experiments again, another member of the lab would be asked to come in and perform the immuno-staining and counts, then the data could be compared to improve reliability.

Another point, which must be considered carefully, are the differences between D6 RNA levels and D6 protein levels observed in this study. While every effort was made to identify where an increase in D6 RNA translated into D6 protein, from this study one cannot conclude this was addressed completely. For example, the 16-fold increase in D6 RNA in uninvolved psoriatic skin was attributed to D6 expression found throughout the viable epidermis, in addition to approximately 95% of all LVs in uninvolved skin. It was suggested that the significant reduction in D6 RNA expression in lesional psoriatic skin compared to uninvolved psoriatic skin was due to the reduction in the percentage of D6 positive LVs in the lesion. However, the major caveat with this is that psoriatic lesions often undergo lymphangiogenesis (Detmar, 2000). Therefore, while uninvolved psoriatic skin has a higher percentage D6 positive LVs compared to lesional psoriatic skin, the total numbers of D6 positive LVs between uninvolved psoriatic skin and lesional psoriatic skin are most likely very similar. This brings one back again to the question of the difference between D6 RNA and D6 protein in uninvolved and lesional psoriatic skin. Therefore, it is clear that an increase in RNA does not always result in an equal increase in the translation of RNA into protein. In the case of D6, it is possible the stability of the RNA or protein, of a particular gene, individually dictates whether an increase in RNA results in an equal increase in protein.

The most obvious question here is, why are virtually all the LVs in uninvolved psoriatic skin D6 positive? From the data in this study we cannot be certain. However, from what we know about the function of D6 on LVs, it is possible to imagine a function for this increase in the percentage of D6 positive LVs. As shown in Figures 3.15. and 3.16., there is detectable expression of inflammatory CC chemokines in uninvolved psoriatic epidermis. It is possible the LVs in uninvolved psoriatic skin 'switch-on' D6 expression in

order to prevent the deposition of inflammatory CC chemokines on the LV (Figure 6.1.A.), which if unchecked, could result in the unwanted binding of inflammatory leukocytes to the LVs (found at elevated numbers in uninvolved psoriatic skin). This binding of inflammatory leukocytes to the LVs could then result in congested lymphatics, and impaired lymphatic function (Figure 6.1.B.), similar to the data reported from inflamed D6 KO mice (Lee et al., 2011).

Therefore in uninvolved psoriatic skin, epidermal-D6 and LV-D6 most likely function in tandem to prevent a build up of inflammatory CC chemokines in uninvolved psoriatic skin, which if unchecked, could result in uncontrolled leukocyte infiltration, which may precipitate lesion formation. Specifically, epidermal-D6 likely forms a barrier restricting and/or blocking inflammatory CC chemokines produced in the epidermis from being released into the dermis. Any epidermal-produced inflammatory CC chemokines which do 'escape' degradation by epidermal-D6, and any dermal produced inflammatory CC chemokines, are prevented from accumulating on the LVs by D6 at this site, which subsequently inhibits the attachment of inflammatory leukocytes to the LVs. Thus D6 on the epidermis and LVs may work in concert to restrict the inflammatory CC chemokine driven immune response.



**Figure 6.1. Proposed function of LV-D6 in uninvolved psoriatic skin (adopted from (Lee et al., 2013))**

A. D6 expression on the surface of the LVs prevents inflammatory CC chemokines from accumulating on the LV surface. Therefore, the inflammatory leukocytes (expressing inflammatory CC chemokine receptors) do not bind to the LV. In contrast, homeostatic CC chemokines (e.g. CCL19) are expressed on the LV surface, facilitating the migration of lymph node homing leukocytes from the skin to the lymph node. B. A loss of D6 expression on the LV surface results in the deposition of inflammatory CC chemokines on the LV surface, which results in aberrant binding of inflammatory leukocytes to the LV surface, impairing the migration of lymph node homing leukocytes from the skin to the lymph node.



#### **6.1.1.4. Leukocyte D6 in psoriasis**

The increase in the number of D6 positive leukocytes in the psoriatic lesion suggests that leukocyte-D6 plays a role in disease pathogenesis. Admittedly, the caveat with this immunostaining is the subjectivity of the counting. As with the D6 positive LV counts discussed in section 6.1.1.3., blinding of samples, in addition to inter-observer checking should have been performed to ensure accurate results, and to prevent possible biasing of the data. In addition, use of LCM to dissect D6 positive leukocytes from the dermis and use of QPCR to examine D6 transcripts could have been used to confirm the increase in D6 positive leukocytes found in the dermis. The most obvious function is the one for which D6 is established, i.e. chemokine-scavenging (Graham, 2009). Leukocytes are motile cells, unlike non-motile D6 expressing cells such the lymphatic endothelial cells of the LVs, and the syncytiotrophoblast cells of the placenta (Martinez de la Torre et al., 2007, Nibbs et al., 2001). Therefore leukocytes can move to the site of inflammatory CC chemokine production during inflammation, and then leukocyte-D6 can degrade the inflammatory CC chemokines at this site, and assist in the resolution of inflammation. However, it has been published previously that co-transfection of cells with D6 and CCR4 inhibits the ability of CCR4 positive cells to migrate towards CCL22, a ligand for both D6 and CCR4 (Bonecchi et al., 2004). Therefore, if leukocyte-D6 functions in a chemokine-scavenging context, it is unlikely the leukocytes would initially express D6 when moving into the skin, as D6 would likely blunt the leukocytes ‘chemotactic potential’. Therefore, it is unlikely that leukocyte-D6 functions primarily in a chemokine-scavenging context.

A second possible function of leukocyte-D6 is that it is responsible for the spatial positioning of leukocytes in the tissue. A recent paper by Graham and colleagues has provided *in vivo* evidence that this could be the case with certain leukocyte populations (Rot et al., 2013). In inflamed D6 KO mice, an accumulation of neutrophils was detected at the dermal/epidermal junction, which caused disruption of the dermal/epidermal junction. Whereas in inflamed WT mice, neutrophils were found in the deeper dermis (Rot et al., 2013). Importantly, this accumulation of neutrophils at the dermal/epidermal junction in inflamed D6 KO mice could be reversed using a CCR1 antagonist (Rot et al., 2013). As CCR1 and D6 bind inflammatory CC chemokines, use of a CCR1 antagonist to block the migration of neutrophils to the dermal/epidermal junction in inflamed D6 KO mice (and subsequent disruption of the dermal/epidermal junction) clearly shows a role for D6 to ensure appropriate spatial positioning of neutrophils in the dermis. Without D6 expression on neutrophils to blunt CCR1 mediated neutrophil chemotaxis by competing with CCR1 for inflammatory CC chemokines, neutrophils migrated to the upper dermis, and caused

disruption of the dermal/epidermal junction. These data suggested that D6 on neutrophils was responsible for preventing the aberrant accumulation of neutrophils in the upper dermis, which caused the detachment of the epidermis from the dermis. Therefore, similar to previous data (Bonecchi et al., 2004), D6 on neutrophils ‘blunted’ the chemotactic potential of neutrophils towards D6 ligands (Rot et al., 2013).

While the data from Graham and colleagues (Rot et al., 2013) suggest a role for D6 in the spatial positioning of leukocytes, how does one incorporate these findings into the data generated in this study? It is possible that leukocyte-D6 in psoriatic lesions is performing a similar function to the role that D6 on neutrophils plays in mice, i.e. ensures the correct spatial positioning of leukocytes in the dermis (Rot et al., 2013). In psoriatic lesions, almost all D6 positive leukocytes were confined to the middle and lower layers of the dermis, and were found to cluster together. Therefore leukocyte-D6 may ensure the various populations of cells are in close proximity to one another, by blunting their chemotactic potential to inflammatory CC chemokines (Figure 6.2.). Of course, care must be taken when attempting to compare *in vivo* mouse work and human pathology data. As the human work is a ‘snap shot’ of what is going on, it is possible that leukocyte-D6 does not have a role in the spatial positioning of leukocytes in the psoriatic lesion. However, from the data we currently have on the function of leukocyte-D6, this is the most likely conclusion.

#### **6.1.1.5. Proposed function of D6 in psoriasis**

Uninvolved psoriatic skin, while histologically normal, has low level, on going inflammation, as shown both in this study and others (see introduction). Inflammatory CC chemokines, responsible for bringing in leukocytes such as T-cells, monocytes and DCs to sites of inflammation, are detectable in uninvolved psoriatic skin, but not in healthy control skin. However, these inflammatory CC chemokines are apparently non-functional, due to the lack of inflammatory lesions in uninvolved psoriatic skin. In addition to detectable inflammatory CC chemokines, D6 RNA expression is significantly elevated in uninvolved psoriatic skin. This increase in D6 RNA expression occurs concomitantly with an increase in D6 protein on the epidermal keratinocytes, and the LVs in the dermis. From the data discussed in this thesis, and the wider literature regarding D6 currently available, the following model is proposed. Epidermal D6 expression in uninvolved psoriatic skin is primarily responsible for preventing the majority of inflammatory CC chemokines produced in the epidermis from being released into the dermal regions as depicted in Figure 6.3.A. As there are modest, but still increased numbers of inflammatory leukocytes in uninvolved psoriatic skin, it is possible that small quantities of inflammatory CC

chemokines do escape D6 mediated degradation as shown in Figure 6.3.A. To prevent these small quantities of inflammatory CC chemokines from accumulating on the LVs, D6 expression is also increased on all the LVs (approximately 95% of all LVs) in uninvolved psoriatic skin, as shown in Figure 6.1.A.

Therefore, uninvolved psoriatic skin is ‘primed’ to develop new lesions as a result of an over-active inflammatory response, which is being kept in check, partially by increase D6 expression. Therefore D6 in uninvolved psoriatic skin, is playing a protective role, limiting the levels of inflammatory CC chemokines available for any possible flares in the inflammatory response, which could result in lesion formation. Similarly, D6 on PBMCs, as suggested previously, is attempting to control increased leukocyte migration into the skin via binding and degrading inflammatory CC chemokines found on the blood vessel endothelial cells. Upon transient loss of D6 expression, e.g. via trauma (See Figure 6.3.B.), inflammatory CC chemokines are released into the dermis, which then start to accumulate on the LVs, which also have transiently lost expression of D6 (the author concedes this was not directly shown via the tape stripping experiment). This transient loss of D6 expression not only results in an increase in the number of leukocytes entering the newly forming lesion, the inflammatory leukocytes themselves now begin to stick to the LVs due to accumulation of inflammatory CC chemokines on the LV surface (See Figure 6.1.B.), thus preventing normal lymphatic drainage, and exacerbating inflammation as shown in mouse model studies (Lee et al 2011). The loss of D6 expression then results in the inflammatory response from ‘taking-over’.

However, the problem in psoriasis is the apparent inability of the immune response to resolve. As shown in the results, D6 expression is found throughout the viable epidermis in uninvolved psoriatic skin and lesional psoriatic skin. It is possible that D6 expression is elevated in uninvolved psoriatic skin, partially via cytokine stimulation, and holds back the inflammatory response from taking hold. However, upon transient loss of D6 expression, the inflammatory response then ensues, and while inflammatory cytokines are likely increasing D6 expression on the epidermis in the lesion, at this stage, D6 is now unable to prevent and/or resolve the inflammatory response. A simple analogy for this would be to think of D6 as a dam holding water back. If the dam is breached, the water will be released, and damage will be done to the surrounding areas. Even if the dam is subsequently repaired, this will not undo the damage already done by the initial release of water, which would then need to be removed by another means. It is therefore likely that the changes in D6 expression observed in psoriasis are a secondary effect of the disease

itself, i.e. a loss of D6 expression occurs after the initial trauma to the skin, which precipitates lesion formation, which then results in a loss of D6 expression to allow inflammation to occur.

### **6.1.2. Discussion of chapter 4 – Regulation of D6 expression**

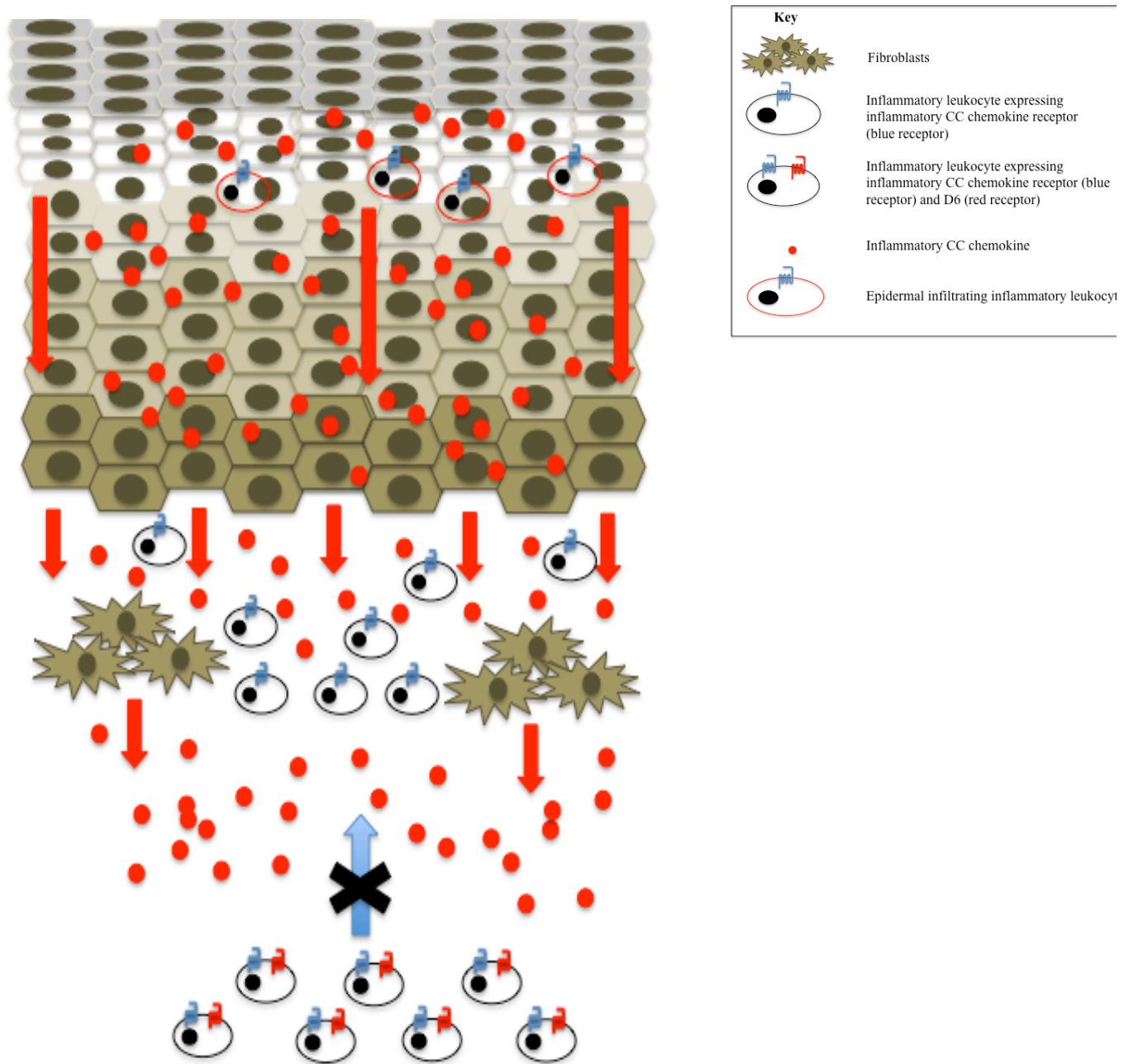
As D6 expression in psoriasis differed markedly depending on the D6 expressing cell type (e.g. LVs, epidermal keratinocytes, leukocytes), or the proximity to the psoriatic lesion (i.e. in uninvolved, peri-lesional, or lesional psoriatic skin) the factors involved in regulating D6 expression were investigated. As there is a 16-fold increase in D6 RNA expression in uninvolved psoriatic skin compared to healthy controls, in addition to the fact that D6 protein was found throughout the viable epidermis and on 95% of all LVs in uninvolved psoriatic skin, primary human epidermal keratinocytes and lymphatic endothelial cells were used to examine factors which could regulate D6 expression *in vitro*. As discussed in the introduction, uninvolved psoriatic skin has on-going, low level inflammation as a number of inflammatory cytokines (Ohta et al., 1991, Uyemura et al., 1993, Yoshinaga et al., 1995), in addition to inflammatory CC chemokines in this study, have been detected at this site. The ability of these inflammatory cytokines detected in uninvolved psoriatic skin to increase D6 expression in primary keratinocyte and lymphatic endothelial cells was therefore tested.

#### **6.1.2.1. Cytokine regulation of D6 expression**

As shown in Figures 4.3.-4.9., cytokine stimulation of D6 expressing cells can increase D6 expression *in vitro*. The rationale for testing IL-6 and IL-1 $\alpha$  was that both cytokines were detected on the epidermis in uninvolved psoriatic skin (Ohta et al., 1991). Stimulating NHEKs or HDLECs with these cytokines gave differing results, which need to be carefully considered. HDLECs stimulated with IL-6/sIL-6R increased D6 expression approximately 8 fold over PBS stimulated controls (McKimmie et al., 2013), in contrast to NHEKs, in which no increase in D6 expression was detected after IL-6/sIL-6R stimulation (Figure 4.3.A.). Similarly, while IL-1 $\alpha$  significantly increased D6 expression in NHEKs (Figure 4.3.B.), stimulation of HDLECs resulted in a reduction in D6 expression compared to PBS stimulated controls (McKimmie et al., 2013). These data suggest differential regulation of D6 by different cytokines, on different D6 expressing cells. It is possible that IL-6 is produced by NHEKs, but does not increase keratinocyte D6 expression *in vivo*. Instead, IL-6 is moved in the interstitial fluid to the LVs where it mediates its effect on D6

expression. It may be that there is subtle control over the regulation of D6 expression at different cutaneous sites.

It is clear that IFN $\gamma$  is a major regulator of cutaneous D6 expression, with striking increases in D6 expression observed in both NHEKs (Figure 4.4.A.) and HDLECs (Figure 4.7.) stimulated with IFN $\gamma$ . A possible cellular source of IFN $\gamma$  in uninvolved psoriatic skin was not identified in this study and this should be addressed. IFN $\gamma$  can be produced by a variety of cell types, but predominantly by NK cells and NK-T cells, in addition to CD4+ and CD8+ T-cells (Thale and Kiderlen, 2005). In one study, significantly more NK cells were detected in uninvolved psoriatic skin compared to healthy control skin (Cameron et al., 2002). It is possible that the IFN $\gamma$  detected in uninvolved psoriatic skin by Nickoloff and colleagues (Uyemura et al., 1993) comes from this population of cells. It is clear from the data that the increase in NHEK-D6 expression can be regulated synergistically by inflammatory cytokines (Figure 4.4.B.). Cytokines involved in psoriasis pathogenesis working synergistically have been shown to function in this manner (Liang et al., 2006). From these data, it is likely that IFN $\gamma$ , TNF $\alpha$ , IL-1 $\beta$ , and IL-17 individually, and in synergy, are responsible for the significant increase in D6 expression in psoriatic skin. While increases in D6 protein were observed in IFN $\gamma$  and IL-6/sIL-6R stimulated HDLECs, in addition to IFN $\beta$  stimulated NHEKs, inter-observer checking, in addition to blinding of studies would have made the experiments more objective. Automated counting would also have been a useful technique to count the samples, as it removes the subjectivity of the individual manually counting the sections. Finally, analyzing the intensity of fluorescence between PBS and cytokine stimulated primary cells would have been another useful technique to test whether an increase in D6 protein was occurring. Similar to automated cell counting, this also takes the subjectivity of the user out of the equation, and thus improving the objectivity of the data.



**Figure 6.2. Proposed model of leukocyte-D6 function.**

Leukocytes expressing inflammatory CC chemokine receptors migrate to the upper dermis, and even into the epidermis itself. However, co-expression of D6 and inflammatory CC chemokine receptors results in the ‘blunting’ of the chemotactic potential for inflammatory CC chemokines.

As shown in Figure 4.5.A. IL-20 and IL-22 do not increase D6 RNA expression. However, it is possible that they may still be responsible for the increase in D6 protein staining in psoriasis lesional keratinocytes. IL-20 and IL-22 are produced by T-cells and DCs, and are involved in inhibiting the differentiation, and inducing the proliferation of, the keratinocyte layer (Boniface et al., 2005). In conjunction with IL-21, which also has a role in the proliferation of the epidermal keratinocytes (Caruso et al., 2009), it is possible that the increased D6 expression on the epidermis in psoriatic lesions is partially due to the effect of these cytokines via expansion of the basal layers of the epidermis. Whether IL-21 can increase D6 expression was not examined in this study, and should be tested in the future.

Type I IFNs (IFN $\alpha$  and IFN $\beta$ ) have been shown to be important for the early stages in lesion formation in psoriasis (Nestle et al., 2005), and mediate their effects through binding to the Type I IFN receptor, which is composed of the receptor subunits IFNAR1 and IFNAR2. IFN $\alpha$  did not increase D6 expression in NHEKs, however IFN $\beta$  stimulation resulted in a two-fold increase in NHEK-D6 expression (Figure 4.5.B.) As both mediate their effects by binding through the type I IFN receptor (Piehler et al., 2012), this was initially a surprising result. However, while IFN $\alpha$  and IFN $\beta$  bind via the same receptor, they have been reported to mediate differing effects (Piehler et al., 2012). In particular, IFN $\beta$  can induce cellular responses such as anti-proliferative activity (Piehler et al., 2012). IFN $\alpha$  can also mediate these effects, but only at very high concentrations (Piehler et al., 2012). In addition, it has been shown that type I IFNs bind to the type I IFN receptor subunits with differing affinities (Piehler et al., 2012). IFN $\beta$  binds to IFNAR1 receptor subunit with at least 20 times higher affinity than any of the other type I IFNs (Piehler et al., 2012). It is possible the ability of IFN $\beta$  to increase D6 expression in NHEKs, in contrast to IFN $\alpha$ , is due to the differential signaling properties of these cytokines. As stated previously, the cytokines used in the cytokine stimulation experiments were used at supraphysiological concentrations. The rationale for this was to ensure the relevant cytokine receptors would be saturated with cytokine, thus ensuring any effect on D6 expression would not be missed as a result of using a small concentration of cytokine. However, the author concedes that at this concentration, higher than would be found in a physiological context, the data must be carefully considered before making any strong conclusions from them.

While the major source of Type I IFNs has been shown in the past to be pDCs, type I IFN production is a response to viral infection, and can be induced in any cell type (Perry et al., 2005) As discussed in the introduction, pDCs have been shown to be activated through a

combination of the antimicrobial peptide LL-37 and genomic DNA in the context of psoriasis (Lande et al., 2007). Keratinocytes in psoriatic lesions have recently been found to produce IFN $\beta$  via sequential activation by LL-37 (which increases TLR9 expression) and CpG motifs (which binds to TLR9). Therefore, both the pDCs, and the keratinocytes themselves may contribute to the production of IFN $\beta$ , which has been shown in this study to increase D6 expression on NHEKs.

#### **6.1.2.2. Correlation analysis of PBMC-D6 expression and inflammatory mediators**

As shown in Figure 4.11., there was no correlation between PBMC-D6 expression and the PASI score. Therefore PBMC-D6 expression cannot be considered as a novel prognostic marker. However in both cohorts of psoriasis patients examined in this study, a significant increase in PBMC-D6 expression was observed (Figures 3.29. and 4.10.). As increased PBMC-D6 expression has also been observed in SSc (Codullo et al., 2011) and Rheumatoid Arthritis (Helen Baldwin, personal communication), it is possible that this elevated PBMC expression of D6 is a general marker of inflammation.

The data from the luminex analysis comparing the plasma levels of inflammatory mediators in healthy controls and psoriasis patients are, in general, consistent with what might be expected in a chronic inflammatory disease (See section 4.4.). Increased psoriasis plasma levels of CCL3 and CCL4 (Figure 4.12.) are consistent with a disease which is considered to be partially mediated by Th1 cells. Both CCL3 and CCL4 bind to CCR5, one of the established Th1 chemokine receptors. Similarly, the increase in psoriasis plasma levels of CXCL8 and CXCL9 (Figure 4.13.) is also logical as psoriasis lesions have neutrophilic infiltration (partially mediated by CXCL8) and an accumulation of Th1 cells (partially mediated via CXCL9). No difference in the levels of circulating CCL11 between healthy controls and psoriasis plasma was perhaps to be expected (Figure 4.12.), as CCL11 has an established role in the recruitment of eosinophils (as discussed in the introduction), which have little role in psoriasis pathogenesis. The modest, but significant reduction of CCL5 in psoriasis plasma compared to healthy controls was unexpected as CCL5 (Figure 4.12.) is associated with Th1 mediated disorders, and is detected in the psoriatic lesions (see results section). Increases in psoriasis plasma levels of IFN $\alpha$  and IFN $\gamma$  (Figure 4.14.) would make sense as pDC produced IFN $\alpha$  is involved in the initiation of psoriasis (Nestle et al., 2005), and IFN $\gamma$  is the signature cytokine of Th1 cells. Again, increased levels of IL-2 in psoriasis plasma compared to healthy controls (as shown in section 4.4.) would be expected as IL-2 is involved in the proliferation of T-cells, and psoriasis is known to be a T-cell mediated disease (Perera et al., 2012). Admittedly, increased IL-4, IL-5 and IL-13



(Th2 cytokines) (See Figure 4.15.) in psoriasis plasma is counterintuitive as psoriasis is not considered to be a Th2 mediated pathology. Increased levels of Th2 cytokines in psoriasis plasma have been published by another group (Deeva et al., 2010), therefore the data in this project are consistent with other findings.

It would not be useful to go through each of the inflammatory mediators measured in the 30 plex and state whether or not this would make sense in accordance with the current literature, as this would very quickly turn into a list of “yes that makes sense”, or “no that does not make sense”. Therefore, a selection of inflammatory mediators was chosen to discuss here as the inflammatory mediators analysed in the 30 plex fell broadly into one of three categories,

1. Chemokines (D6 binding and non-D6 binding)
2. Cytokines/Growth factors associated with psoriasis pathogenesis (e.g. IFN $\alpha$  and IFN $\gamma$ )
3. Cytokines/Growth factors not normally associated with psoriasis pathogenesis (e.g. IL-4, 5 and 13).

The more fundamental question to be asked, is what are these inflammatory mediators doing in the plasma? From the literature, there does not appear to be an exact answer to these questions. The simplest answer to this question is that inflammatory mediators in the circulation were produced in the peripheral tissue, e.g. the skin, then the interstitial fluid containing these inflammatory mediators moves into the lymphatic vessels, and ultimately ends up in the circulation. Alternatively it is possible that leukocytes in the plasma, or the blood vessel endothelial cells, produce the inflammatory mediators. A lot of the cytokines and chemokines increased in psoriasis plasma compared to healthy controls would fit with the profile of a chronic inflammatory Th1/17 mediated illness.

The positive correlation between PBMC-D6 and CCL4 (Figure 4.18.) is interesting, as one might expect a negative correlation, i.e. the higher the levels of PBMC-D6, the more D6 can internalize CCL4, therefore reduced CCL4 levels. However it is possible that PBMC-D6 and CCL4 in the plasma are working together to reduce the number of PBMCs entering the skin. Specifically, elevated CCL4 in the plasma will result in down-regulation of inflammatory CC chemokine receptors on PBMCs, reducing the ability of the PBMCs to bind to inflammatory CC chemokines on the lumen of the blood vessel endothelial cells. D6 on PBMCs will compliment this effect by ensuring that PBMCs which do start to bind to the lumen of the blood vessel via interaction with inflammatory CC chemokines

immobilized on the vessel walls will degrade the inflammatory CC chemokines, thus allowing the PBMC to move back into the plasma.

The increase in PBMC-D6 expression in cells cultured with increasing concentrations of serum suggests that there is a factor in the serum which can regulate PBMC-D6 (Figure 4.23.) As shown in Figure 4.25., lipids can regulate D6 expression, as removal of lipids from the serum resulted in a significant reduction in PBMC-D6 transcript numbers. However, as no difference in the levels of PBMC-D6 transcript numbers was observed when PBMCs were stimulated with either healthy control plasma or psoriasis plasma (Figure 4.24.), it is unlikely that a lipid is responsible for the increase in psoriasis PBMC-D6 transcripts. It is also possible that the increased PBMC-D6 expression is due to the increase of D6 expression in a minor population of D6 expressing leukocytes. Increased numbers of Th1, Th17 and Th22 cells have been detected in the circulation of psoriasis patients (Kagami et al., 2010). It is possible the increase in D6 expression in psoriatic PBMCs is due to elevated D6 expression in one or all of these populations.

#### **6.1.2.3. The effect of trauma on D6 expression**

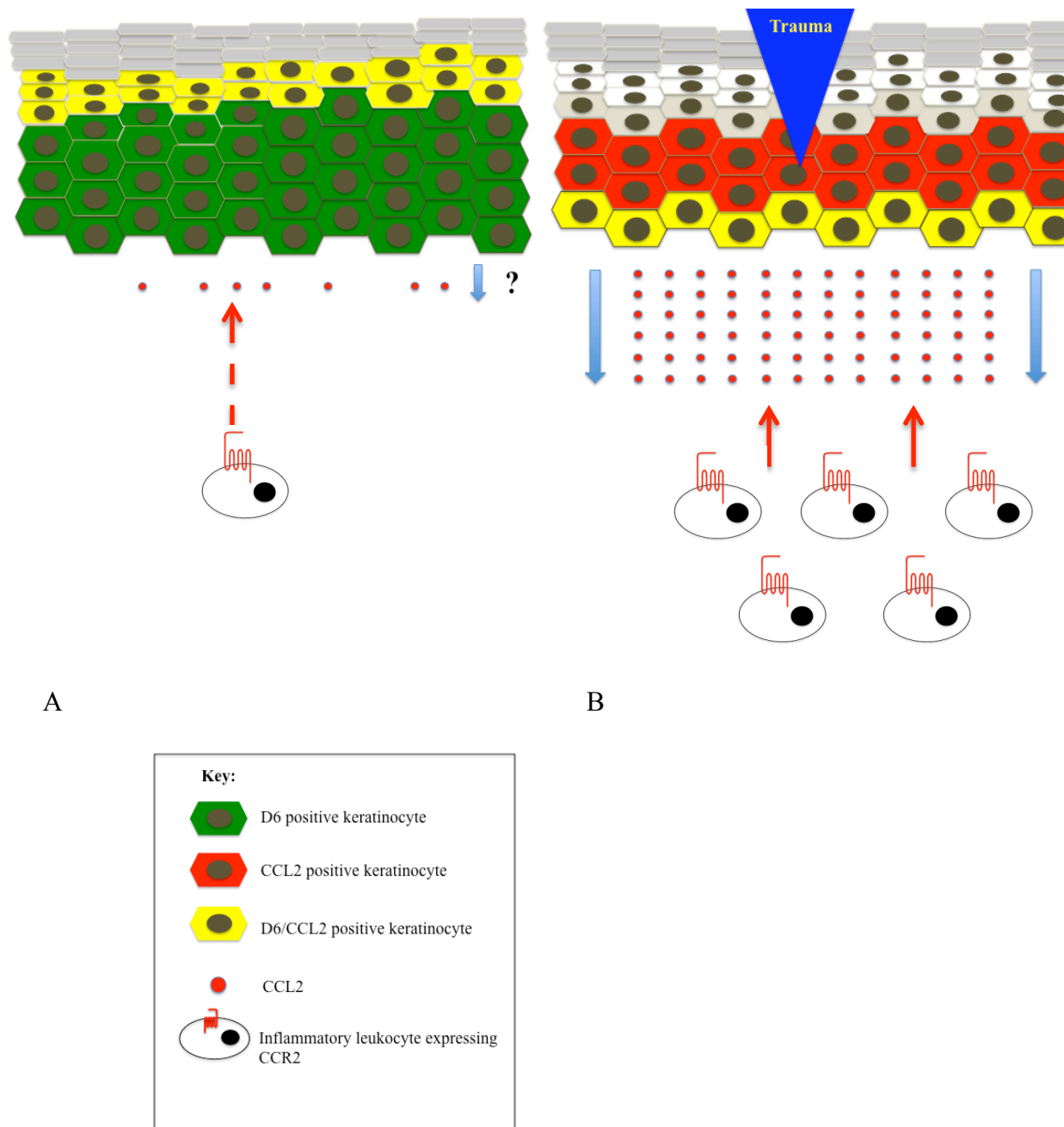
The reduction in D6 expression on the epidermis after micro-trauma to uninvolved psoriatic skin (Figure 4.26.) suggests a mechanism to explain how D6 expression is reduced when comparing uninvolved psoriatic skin and peri-lesional psoriatic skin. As psoriasis lesions tend to occur at sites that are prone to continuous micro-trauma, e.g. the elbow, knees and scalp (Perera et al., 2012), a loss of D6 expression at the site of trauma is logical. The loss of D6 expression in the peri-lesional skin is likely to result in a transient loss in the function of D6, resulting in the release of inflammatory CC chemokines into the dermis (Figure 6.3.). The inflammatory response can then ‘gain a foothold’ and lesion formation can ensue. As uninvolved psoriatic skin is ‘inflammatory’ in nature, there is a ready-made supply of inflammatory cytokines and chemokines at this location. Uninvolved psoriatic skin therefore can be considered to be ‘primed’ for the development of new lesions.

While a reduction of epidermal-D6 expression does occur upon micro-trauma to uninvolved psoriatic skin (Figure 3.2.6.), no follow-up study was performed. Therefore, it cannot be said with absolute certainty that micro-trauma to the patient’s skin results in the development of new lesions. In addition, micro-trauma did not reduce the percentage of D6 positive LVs in traumatised (previously uninvolved) psoriatic skin (Figure 4.26.D.). This is important as in peri-lesional psoriatic skin, a significant reduction in D6 expression is

observed both on the epidermis (Figures 3.9.-3.12.), and in the percentage of D6 positive LVs compared to uninvolved psoriatic skin (Figures 3.20. and 3.22.). Therefore a reduction in the percentage of D6 positive LVs appears to be important for the development of new lesions.

#### **6.1.2.4. Mouse models of psoriasiform pathology**

Mouse models of psoriasiform pathology were used to analyse whether D6 expression in these models mirrored D6 expression in clinical samples. If D6 expression in these models did match D6 expression in clinical samples, they would allow an in depth analysis of the molecular mechanism of regulation of D6 expression in psoriasis *in vivo*. For example, if cytokines found in uninvolved psoriatic skin are responsible for the elevation of D6 expression at this site, how exactly are the cytokines produced at this location? One possibility is that cells in the uninvolved psoriatic epidermis produce the cytokines. Alternatively, it is possible that the cytokines are produced in the psoriatic lesion, leave this site in the lymph, enter the circulation, and eventually end up in uninvolved psoriatic skin.



**Figure 6.3. Proposed mechanism of leukocyte infiltration into the dermis.**

A. In uninvolved psoriatic skin, D6 is found throughout the epidermis (green keratinocytes) and co-stains with CCL2 (yellow keratinocytes). The question mark denotes the possibility that small quantities of inflammatory CC chemokines (red circles), may be escaping degradation by epidermal-D6. The broken red arrow denotes weak, but detectable chemo-attraction for the low numbers of infiltrating leukocytes in uninvolved psoriatic skin. B. Uninvolved psoriatic skin that has undergone micro-trauma. This results in a loss epidermal D6 expression, which results in the release of inflammatory CC chemokines (red circles), and subsequent infiltration of the previously uninvolved psoriatic skin with a large population of leukocytes. D6 expression is now localised to the lowest level of the epidermis and co-stains with CCL2 (yellow keratinocytes). However, this reduced D6 expression is not sufficient to prevent the release of inflammatory CC chemokines into the dermis.

In both mouse models used in this study (S100a7/S100a15 double transgenic mouse model and the Imiquimod mouse model), a reduction in D6 expression was observed in the skin lesion compared to a site distant (similar to uninvolved psoriatic skin) from the lesion. D6 was originally associated with psoriasis pathogenesis when a D6 KO mouse, treated with phorbol ester to the shaved dorsal skin, developed an exaggerated, and prolonged inflammatory skin pathology, similar in many respects to psoriasis (Jamieson et al., 2005). In contrast, application of phorbol ester to the shaved dorsal skin of WT mice resulted in a minor inflammatory reaction, which quickly resolved (Jamieson et al., 2005). Therefore, a loss of D6 expression appeared to ‘prime’ the mouse, or increase the susceptibility of the mouse, to developing a cutaneous inflammatory pathology that resembles psoriasis. In the mouse model developed by Yuspa and colleagues, over-expression of the S100 proteins S100a7 and S100a15 in the epidermis resulted in no obvious resting phenotype (Wolf et al., 2010). However, and similar to the D6 KO mouse, application of TPA to the shaved dorsal skin of S100a7/S100a15 double transgenic mice resulted in cutaneous inflammation, resembling psoriasis (Wolf et al., 2010). Therefore, genetic manipulation resulted in the S100a7/S100a15 double transgenic mouse becoming ‘primed’ to develop a psoriasiform pathology. While these models cannot be directly compared as one made use of gene deletion, and another over-expressed two genes, the one consistency with both data sets is that a reduction in D6 expression was associated with a psoriasiform pathology. In the D6 KO mouse, it is of course a global deletion of D6. In the S100a7/S100a15 double transgenic mouse, a reduction in D6 expression is localized to the site of lesion formation. The Imiquimod mouse model of psoriasiform pathology also showed that reduced D6 expression occurred at the site of lesion formation. Therefore these mouse models in some ways, mirror the expression of D6 in psoriasis pathogenesis.

Of course, no mouse model replicates all the features of human psoriasis, as stated in the introduction. In both psoriatic skin, and the mouse models of psoriasiform pathology used in this study, a significant reduction in D6 expression occurs in the skin lesion, compared to a site distant from the skin lesion. However, at the site distant from the skin lesion in psoriatic patients (i.e. uninvolved psoriatic skin), D6 expression is significantly higher compared to healthy control skin. In contrast, D6 expression at the site distant from the lesion in mice is similar to Vaseline stimulated mice (equivalent to the healthy control skin). Therefore in psoriasis, D6 expression in uninvolved psoriatic skin is significantly elevated compared to healthy control skin, and lesional psoriatic skin, suggesting a pro-active action of the body to inhibit lesion formation at non-lesional sites. In the mouse models used, no equivalent increase in D6 expression is observed.

### **6.1.3. Discussion of chapter 5 – D6 in eczema**

#### **6.1.3.1. D6 RNA expression in eczema**

Analysis of eczema skin allowed the comparison of D6 expression in a second cutaneous inflammatory pathology. Importantly, as psoriasis is a Th1/Th17/Th22 inflammatory disease, whereas eczema is predominantly Th2 biased, certainly during the initial phases of the disease, it allowed an examination of cutaneous D6 expression in a different inflammatory context. No significant increase in D6 RNA expression was observed in eczema lesions compared to healthy controls (Figure 5.1.). These data suggest fundamental differences in the pathology of psoriasis and eczema.

#### **6.1.3.2. Epidermal-D6 in eczema**

Examination of D6 protein expression in eczema lesions found that, similar to psoriatic lesions, an increase in D6 expression on the epidermal keratinocytes was observed compared to healthy controls (Figure 5.2.). However, D6 expression in eczema keratinocytes was found towards the lower layers of the epidermis, whereas D6 in psoriasis lesional keratinocytes was found throughout the viable epidermis. The simplest explanation for this is that as psoriasis is a disease where Th1/Th17/Th22 cells predominate, the cytokine profile will consist of cytokines such as IFN $\gamma$ , TNF $\alpha$  and IL-1 $\beta$ , all of which have been shown to increase D6 expression on NHEKs *in vitro*. Whereas eczema is a Th2 mediated disease initially, and the Th2 cytokines IL-4, IL-5 and IL-13 have been shown to have no effect on D6 expression *in vitro*. (Ruairidh Nicoll, Mark Singh and Gerard Graham, unpublished observations). Therefore, keratinocytes in psoriatic lesions will be exposed to Th1 cytokines throughout disease pathology, whereas eczema lesional keratinocytes will have a reduced exposure to Th1 cytokines, which may help to explain the reduced D6 immuno-reactivity on the epidermis compared to psoriatic lesional keratinocytes.

#### **6.1.3.3. LV-D6 in eczema**

Similar to the data observed in psoriasis lesions, no significant increase in the percentage of D6 positive LVs in eczema skin was observed compared to healthy control skin (Figure 5.3.). However, no assumptions can be made about these data, as unlike in psoriasis, no uninvolved skin from eczema patients was examined as we were unable to obtain biopsies from uninvolved eczema skin. In addition, eczema lesions are not as well demarcated as psoriatic lesions. It is possible therefore that D6 expression is elevated at a site distant from

the eczema lesion, similar to the psoriatic lesion. Similar to D6 positive LVs studied in psoriasis, these data should have been confirmed using the additional experiments suggested in section 6.1.1.3.

#### **6.1.3.4. Leukocyte-D6, and inflammatory CC chemokine expression in eczema**

A significant increase in the number of D6 positive leukocytes was observed in eczema lesions compared to healthy controls (Figure 5.5.) (similar to the increase in D6 positive leukocytes in psoriatic lesions). It is possible that D6 has a role in either the spatial positioning of the leukocytes in the dermal regions, or in the scavenging of inflammatory CC chemokines in the lesion, equivalent to the role leukocyte-D6 was proposed to have in psoriasis pathogenesis (see section 6.1.1.4.). Co-staining for D6 and CCL2 or CCL5 showed a similar pattern of distribution as in psoriatic epidermis (Figures 5.9. and 5.10.). Specifically, D6 expression was strongest towards the lower layers of the epidermis, whereas CCL2 and CCL5 expression predominated towards the upper layers of the epidermis. These data reinforce the idea that D6 on the epidermis acts as a barrier to unrestricted inflammatory CC chemokine release into the dermis.

To sum up, in this study D6 expression was found to be elevated in both psoriasis and eczema, albeit the increase in D6 expression was more pronounced in psoriasis. These data suggest that D6 may be a possible target in a therapeutic context. However, exactly how D6 can be manipulated to improve disease pathogenesis remains to be determined.

#### **6.1.4. Future work**

With the basic D6 expression data generated in this study, further studies are now required for a more in depth analysis of D6 function in psoriasis. In this study, a reduction in D6 expression occurred in the peri-lesional psoriatic skin relative to uninvolved psoriatic skin, suggesting that a reduction of D6 expression is sufficient to induce lesion formation. In addition, micro-trauma to uninvolved psoriatic skin resulted in a significant reduction in D6 expression. As psoriatic patients have a greater propensity to develop new lesions at previously uninvolved sites via the Koebner phenomenon (Weiss et al., 2002), it is possible a loss of D6 expression is an early event in this process. However, this was never formally tested. Specifically, no follow-up checks were performed on the traumatised patient's skin to determine whether lesion formation did occur, and how D6 expression was regulated in this context. Therefore, from this study, we cannot definitively say that a loss of D6 expression is a pre-requisite for lesion formation. To formally test this, tape stripping of uninvolved psoriatic skin should be performed as before, followed by taking

sequential biopsies 24 hours after injury, in addition to a biopsy every week for 3-4 weeks post-trauma. In addition, a biopsy from the same patient should be taken from an existing chronic plaque to compare the development of the lesion against existing lesions. This may seem very extreme, but it has been performed previously (Raychaudhuri et al., 2008), and provided unique insights into the development of the psoriatic lesion. As shown in chapter 4 of this thesis, a reduction of D6 expression in traumatised (previously uninvolved psoriatic skin) was localised to the epidermis, but not the LVs. It is possible that for Koebnerization to occur, a loss of D6 expression needs to occur simultaneously in the keratinocytes and the LVs. Alternatively, it may be that a loss of D6 expression on the LVs occurs later than 24 hours post trauma.

Sequential biopsies would also allow an examination of D6 positive leukocytes in the developing lesion. As shown by Graham and colleagues, leukocyte-D6 has a role in the spatial positioning of neutrophils in the dermis, and blunts the chemotactic potential of the neutrophils to its ligands (Rot et al., 2013). Use of sequential biopsies would allow a study of whether leukocyte-D6 expression regulates the position of leukocytes in psoriatic skin. Specifically, D6/CD45 co-staining should be performed, then the location of D6 positive leukocytes and D6 negative leukocytes in the tissue should be analysed. It is possible that the D6 positive leukocytes would be found in the lower dermis, whereas D6 negative leukocytes would be detected in the upper dermis, towards the dermal-epidermal junction.

Another area of D6 research that needs examining is the relationship of microRNAs and D6, and the role of microRNAs have (if any) in regulating D6 expression in psoriasis. microRNAs regulate genes at the post-transcriptional level, and are increasingly being looked at as a novel therapeutic target (Schneider, 2012). Preliminary data from our laboratory suggest that microRNAs, which are elevated in psoriatic lesions, can significantly reduce D6 expression in NHEKs and HDLECs (Kave Shams, personal communication). It is possible that microRNAs are partially responsible for the significant reduction in D6 expression in lesional psoriatic skin compared to uninvolved psoriatic skin. Therefore these microRNAs could be targeted in an attempt to restore D6 expression in lesional psoriatic skin to levels observed in uninvolved psoriatic skin. To test this in a more functionally relevant setting, the xenograft mouse model of psoriasis (Boyman et al., 2004). (discussed in section 1.6.2.4.) could be used. microRNAs which are over-expressed in psoriatic lesions, and have been shown to reduce D6 expression *in vitro*, could be targeted using antagomirs (nucleotides designed to block microRNA function by binding to the 3' untranslated region the microRNA is specific for) (Schneider, 2012). It is possible



that blocking microRNAs specific for D6 could result in increased D6 expression, and a reduction in inflammation.

Recently there has been an interest in microRNAs in plasma as biomarkers (Ceman and Saugstad, 2011). At present there is little data on the potential of microRNAs as biomarkers in the context of psoriasis (Only 4 articles come up on Pubmed by typing 'psoriasis and biomarker and microRNA'). As we currently have surplus plasma from psoriatic patients, in addition to healthy control samples, this would be an excellent opportunity to search for novel markers of disease severity. Within the context of D6, as PBMC-D6 expression is significantly elevated in psoriatic patients, it is possible that there is a significant reduction in microRNAs specific for degrading PBMC-D6.

Another question, which should be investigated, further is whether the IFN $\gamma$  signaling pathway can be used to increase D6 expression in psoriatic lesions, in an attempt to reduce inflammation. Stimulation of HDLECs induced a 9-fold increase in D6 expression 6 hours post stimulation, suggesting this rapid and striking increase in D6 expression is a primary effect of the cytokine. As the percentage of D6 positive LVs is significantly reduced in lesional psoriatic skin compared to uninvolved psoriatic skin, manipulation of this pathway could possibly 'switch-on' LV-D6, which might reduce inflammation in the skin. Of course, injecting psoriasis patients with IFN $\gamma$  has been shown previously to exacerbate the pathology (Fierlbeck et al., 1990). Therefore, an analysis of the IFN $\gamma$  signaling networks (Hu and Ivashkiv, 2009) could yield potential molecules which could be tested to see whether they could increase D6 expression *in vitro* using HDLECs. If candidate molecules were shown to increase D6 expression *in vitro*, these molecules could be tested in a more functionally relevant setting. Specifically, the xenograft model of psoriasis could be used again, and mice injected with candidate molecules in an attempt to increase D6 expression, and attenuate the levels of inflammation.

In conclusion, from this work it is clear that D6 is elevated in cutaneous disease, possibly in an attempt to inhibit the inflammatory CC chemokine driven immune response. The next challenge is to harness the ability of D6 to scavenge inflammatory CC chemokines in a therapeutic context.

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